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**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

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| Application of: | Chen et al. | Confirmation No.: | 6007 |
| Serial No.: | 09/992,860 | Art Unit: | 1654 |
| Filed: | November 14, 2001 | Examiner: | Michele C. Flood |
| For: | Black Tea Extract For Prevention Of Disease | Attorney Docket No.: | 11592-025-999 (formerly RU-0173) |

**DECLARATION OF DR. CHI-TANG HO UNDER 37 C.F.R. § 1.132**

Commissioner for Patents
Washington, D.C. 20231

Sir:

I, CHI-TANG HO, do declare and state that:

1. I am a citizen of the United States residing at 32 Jernee Drive, East Brunswick, New Jersey 08816.

2. I am one of the inventors of the invention described and claimed in the above-identified patent application, Serial No. 09/992,860, (the "'860 application").

3. I received the degree of Doctor of Philosophy from Washington University in 1974. I am an author in over 300 refereed scientific papers and over 160 book chapters.

4. Since 1978, I have been employed by Rutgers University, the assignee of this application. In particular, since 1978, I have been a Full Member of the Graduate Program in Food Science, at Rutgers University and became the Director in 2003. Since 2002, I have also been a Full Member of the Graduate Program in Medicinal Chemistry, at the College of Pharmacy, Rutgers University.

5. My professional experiences and honors, and a list of my publications, patents, and Ph.D. theses I have advised are set forth in my *curriculum vitae,* attached hereto as Exhibit 1.

6. Effects of TF-2 on Cancer Cells

6.1 The experimental results presented at pages 3 to 6 of the '860 application demonstrate that the theaflavin monogallates, theaflavin-3-gallate and theaflavin-3'-gallate, (TF-2) exhibit significant differential inhibiting effects on cancer cells. In addition, I am a coauthor of the publication, Lu *et al.* (2000, Cancer Research, 60:6465-71, "Lu"), which is attached hereto as Exhibit 2. The studies described therein were conducted by myself and my collaborators, and I am familiar with the results. The results in Lu in large part reiterate the results presented in the '860 application, including graphical representation of such results.

6.2 Cell growth inhibition studies

The results presented in the '860 application demonstrate that TF-2 exhibits a differential growth inhibiting effect on two different art-accepted cancer cell lines.

First, growth of cancerous fibroblast cells (WI38VA) was inhibited significantly by TF-2 (but not the other two theaflavin polyphenols, TF-1 and TF-3) while there was no significant effect on the growth of normal diploid fibroblast cells (WI38)(see page 3 of the specification, lines 29-31). See, also, Figure 3A and 3B of Lu on page 6467 which shows the differential effect of TF-2 on the growth of W138 cells vagues WI38VA cells. Lu estimated that there is a difference of two orders of magnitude in the $IC_{50}$ value of TF-2 towards WI38 cells versus WI38A cells (see page 6466, second column, second full paragraph, lines 18-21).

The inhibitory effect of TF-2 was further tested with Caco-2 colorectal cancer cells and normal CCD-33Co cells as described on page 4 of the specification, lines 10-13. Lu reported the same results graphically in Figure 4 at page 6467 and included the results obtained with a normal human colon cell strain (FHC). TF-2 at 50μM inhibited the growth of Caco-2 cells but had little effect on the growth of

CCD-33Co or FHC cells. Neither of the other two theaflavin polyphenols, TF-1 and TF-3, exhibited a differential growth inhibitory effect.

6.3 Apoptosis Induction

The results presented in the '860 application also demonstrate that TF-2 differentially induces apoptotic cell death in transformed cells.

The differential effects of TF-2 on the induction of apoptosis in normal cells (WI38) versus transformed, cancerous cells (WI38VA) are demonstrated by a TUNEL assay as described in the specification on page 4 at lines 24-28. See also, Figure 5 of page 6468 of Lu. In a TUNEL assay, apoptotic cells are detected by labeling with fluorescein-12-dUTP. Here, TF-2 caused almost all WI38VA cancer cells to become fluorescent while none of the TF-2-treated WI38 normal cells were labeled.

Endonuclease fragmentation of nuclear DNA into small DNA fragments is a hallmark of apoptosis in many cell types. The results of a DNA fragmentation analysis show that TF-2 caused fragmentation in WI38VA cells within four hours of TF-2 treatment while there was no significant fragmentation in WI38 cells over a 24 hour period (see specification on page 4, lines 30-33). See, also, Figure 6, page 6468 of Lu.

These data sets show that TF-2 differntially induces apoptotic in cancerous cells. Without wishing to be bound by any particular theory or mechanism of action, these results can explain, at least in part, the differential effect of TF-2 on normal and cancerous cells.

6.3 A direct link between cox-2 gene expression level and polyps formation has been demonstrated in Apc knockout mice, an art-accepted model for colorectal cancer, indicating a direct role of cox-2 in colon cancer formation (see

Oshima et al., 1996, Cell 87:803-809 as referred to in the specification at page 5, lines 5-20). As shown in Lu, TF-2 specifically inhibits cox-2 gene expression.

The effect of TF-2 on cox-2 gene expression in cells from a colon cancer cell line (Caco-2) is shown on pages 6468-6469 and Figure 7 of Lu. TF-2 at 50-100 μM prominently suppressed both serum induced and basal cox-2 gene expression in Caco-2 cells. The effects of TF-2 on cox-2 gene expression in WI38 and WI38VA cells are shown on pages 6468-6469 and Figure 8 of Lu. TF-2 blocked the serum-induced cox-2 gene expression in both WI38 and WI38A cells. Pages 6469-6470 and Figure 9 of Lu show that the dramatic effect of TF-2 on the expression of cox-2 is a specific effect, in that cox-2 is the only growth-related gene tested whose expression is dramatically attenuated by TF-2.

6.4 In view of the foregoing, I conclude, and it is my opinion that others skilled in the art would also conclude that, based on the differential effects of TF-2 on normal and cancer cells and the specific effect of TF-2 on cox-2 gene expression, TF-2 is useful for the treatment and prevention of colorectal cancer.

7. Effect of Black Tea Extract In A Mouse Model of Colorectal Cancer

7.1 I and others are coauthors of a chapter in a book entitled "Herbs: Challenges in Chemistry and Biology", ACS Symposium 925, ed. by Wang et al., published by American Chemical Society, Washington, D.C., 2005. The chapter title is "Effect of black tea theaflavins in 12-O-tetradecanoylphorbol-13-acetate-induced inflammation, expression of pro-inflammatory cytokines, arachidonic acid metabolism in mouse ear and colon carcinogenesis in Min (Apc+/-) mice" ("Huang"; attached hereto as Exhibit 3). The study described therein was conducted by myself and my collaborators, and I am familiar with the results. The study was designed to assess the effects of black tea theaflavins on, *inter alia*, spontaneous

carcinogenesis in the intestine of mice. The results presented in Huang corroborate the teachings presented in the '860 application that TF-2 can be used to treat and/or prevent colorectal cancer.

7.2 Min mice (heterozygote for the adenomatosis polyposis coli gene, *Apc*+/*Apc*-), which were used in the experiments described in the manuscript of Exhibit 3, is a well-established animal model of human colorectal cancer and cancer formation.

In both mice and humans, this mutation in the *Apc* gene leads to early development of intestinal adenomas that can progress to carcinomas. A description of the mouse strain and a list of publications describing the uses of this mouse strain, obtainable from the supplier, Jackson Laboratory, are attached hereto as Exhibit 4.

7.3 In a study of the gene expression profiles of intestinal tissues of Min mice and human cancer patients described in Chen et al. (2004, Cancer Research, 64:3694-3700, attached hereto as Exhibit 5), cox-2 gene expression is shown to be up-regulated significantly in the adenomatous polyps (a precursor of cancer) and mucosa of Min mice, as well as in colon samples of human colon cancer patients. These expression data show that the Min mouse is an appropriate model for testing compounds inhibitory to cox-2 gene expression in human colorectal cancer tissues.

7.4 Huang demonstrates that oral administration of 0.2% and 0.4% of black tea extract (28% theaflavins) in drinking water as the sole source of drinking fluid for 10 weeks inhibits the formation of colorectal tumors per mouse by 59% or 34%, respectively. The percent of mice bearing colorectal tumors was inhibited by 56% or 29%, respectively (see Huang, Figure 4 of Exhibit 3).

7.5 Huang also demonstrates that oral administration of 0.2% and 0.4% of black tea extract inhibits formation of small intestinal tumors per mouse by 39%

and 29%, respectively. The total small and large intestinal tumors per mouse were inhibited by 39 and 29%, respectively (see Figure 5 of Exhibit 3).

7.6 The *in vivo* results described in ¶¶ 7.4 and 7.5 correlate with the results observed in cell-based, *in vitro* experiments, and demonstrate the inhibitory effect of theaflavins, including theaflavin-3 gallate and theaflavin-3' gallate, on the formation of adenomas and carcinomas in a mouse model of colon cancer that is a well-established animal model for human colorectal cancer.

7.7 In view of the foregoing, I conclude, and it is my opinion that others skilled in the art would also conclude that, theaflavin-3 gallate and theaflavin-3' gallate can be used successfully to treat and prevent colorectal adenomas as well as colorectal cancer in humans.

8. I declare further that all statements made in this Declaration of my own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated: June 19 2006

Chi-Tang Ho

CHI-TANG HO

Attachments:

Exhibit 1: *Curriculum Vitae* of Chi-Tang Ho

Exhibit 2: Lu et al., 2000, "Differential Effects of Theaflavin Monogallates on Cell Growth, Apoptosis, and *Cox-2* Gene Expression in Cancerous *versus* Normal Cells", Cancer Research 60:6465-6471.

Exhibit 3: Manuscript entitled "Effect of black tea theaflavins in 12-O-tetradecanoylphorbol-13-acetate-induced inflammation, expression of pro-inflammatory cytokines, arachidonic acid metabolism in mouse ear and colon carcinogenesis in Min (Apc+/-) mice" by Huang et al.

Exhibit 4: Print-out of JAX Mice Data Sheet and a list of additional references relating to Min mouse available on the web site of The Jackson Laboratory 600 Main Street, Bar Harbor, Maine 04609 USA.

Exhibit 5: Chen et al., 2004, "Alteration of Gene Expression in Normal-appearing Colon Mucosa of $APC^{min}$ Mice and Human Cancer Patients", Cancer Research, 64:3694-3700.

# Curriculum Vitae of Chi-Tang Ho

NAME: Chi-Tang Ho

PRESENT TITLE: Professor II and Graduate Program Director

DEPARTMENT: Department of Food Science
ADDRESS: Cook College, Rutgers, The State University
65 Dudley Road, New Brunswick, NJ 08901–8520

EDUCATION:

B.S. (Chemistry). National Taiwan University. 1968.
M.A. (Organic Chemistry). Washington University. 1971.
Ph.D. (Organic Chemistry). Washington University. 1974.

PROFESSIONAL EXPERIENCE:

1975–76 Postdoctoral Fellow – School of Chemistry, Rutgers University
1976 Postdoctoral Fellow – Department of Food Science, Rutgers University
1976–78 Assistant Research Professor – Department of Food Science, Rutgers University
1978-83 Assistant Professor, Department of Food Science, Rutgers University
1978–present Full Member, Graduate Program in Food Science, Rutgers University
1983–87 Associate Research Professor, Department of Food Science, Rutgers University
1987–93 Professor I, Department of Food Science, Rutgers University
1993–present Professor II, Department of Food Science, Rutgers University
1993–present Cluster Coordinator, Basic Research Program, Center for Advanced Food Technology, Rutgers University
1996–present Faculty Member, Environmental and Occupational Health Sciences Institute, Rutgers University
2002–present Full Member, Graduate Program in Medicinal Chemistry, College of Pharmacy, Rutgers University
2003–present Director, Graduate Program in Food Science, Rutgers University
2003–present Member, Cancer Institute of New Jersey, UMDNJ-Robert Wood Johnson Medical School

HONORS AND AWARDS:

ACS Award for the Advancement of Application of Agricultural and Food Chemistry: American Chemical Society, 2005.
Elected Fellow: Institute of Food Technologists, 2003.
IFT Stephen S. Chang Award for Lipid or Flavor Science: Institute of Food Technologists, 2002.
Board of Trustees Award for Excellence in Research at Rutgers, The State University of New Jersey: Rutgers University, May 2, 2002.
Cited as "Highly Cited Researcher" in Agricultural Sciences: Institute for Scientific Information, 2002.
Spotlight Award for Research Excellence: Cook College, Rutgers University, April 1998.
Fellow Award: Agricultural and Food Chemistry Division, American Chemical Society, September 1988.
American Chemical Society Newsmaker for Research in an ACS Journal Award, August 2002.
Distinguished Service Award: Agricultural and Food Chemistry Division, American Chemical Society, August 2000.
Honorary Professor: Wuxi Institute of Light Industry, Wuxi, The People's Republic of China, 1988 – 2003.
Honor Professor and Doctoral Supervisor: Southern Yangtzi University, Wuxi, The People's Republic of China, 2003 – present.
Honorary Professor: China Agricultural University, Beijing, The People's Republic of China, 1988–present.
Honorary Professor: Anhui Agricultural University, Hefei, Anhui, The People's Republic of China, 2000–present.
Platinum Award: Agricultural and Food Chemistry Division, American Chemical Society, August 1994 & August 1995.
Associate Editor: *Journal of Food Science*, 2002–present.
Advisory Board Member: *Journal of Agricultural and Food Chemistry*, 1995–2006.
Editorial Board Member: *Food Reviews International*, 1987–present.
Editorial Advisory Board Member: *Critical Reviews in Food Science and Nutrition*, 1987–present.
Editorial Board Member: *Journal of Food Lipids*, 1996–present.
Editorial Advisory Board Member: *Bailey's Industrial Oil & Fat Products*, Fifth Edition, 1994–1996; and Sixth Edition, 1999–2004.
Chair: Division of Agricultural and Food Chemistry, American Chemical Society, 1996.
Chair: Nutraceuticals and Functional Foods Division, Institute of Food Technologists, 2003–2004.
Editorial Board Member: *BioFactors*, 2004–present. .
Senior Editor: *Molecular Nutrition and Food Research* 2004–present.

**List of Publications:**

## *1. Refereed Publications*

1) Gaspar, Peter P.; Ho, Chi-Tang; Choo, Kwang Yul. Organosilicon iminamino radicals from the addition of silyl radicals to diphenyldiazomethane. *Journal of the American Chemical Society* 1974, *96*, 7818–7820.
2) Ho, Chi-Tang; Conlin, Robert T.; Gaspar, Peter P. A hydrocarbon catalyst for diazoalkane decomposition. *Journal of the American Chemical Society* 1974, *96*, 8109–8111.
3) Moss, Robert A.; Ho, Chi-Tang. 7-Norbornenylidene - stereoselectivity of the addition reaction as a test for bridge-bending. *Tetrahedron Letters* 1976, 1651–1654.
4) Moss, Robert A.; Ho, Chi-Tang. 2-Methyl-7-norbornenylidene - the methyl probe for homoallylic delocalization. *Tetrahedron Letters* 1976, 3397–3400.
5) Moss, Robert A.; Mallon, Charles B.; Ho, Chi-Tang. The correlation of carbenic reactivity. *Journal of the American Chemical Society* 1977, *99*, 4105–4111.
6) Ho, Chi-Tang; Smagula, Michael S.; Chang, Stephen S. The synthesis of 2-(1-pentenyl)furan and its relationship to the reversion flavor of soybean oil. *Journal of the American Oil Chemists' Society* 1978, *55*, 233–237.
7) Chang, Stephen S.; Peterson, Robert J.; Ho, Chi-Tang. Chemical reactions involved in the deep-fat frying of foods. *Journal of the American Oil Chemists' Society* 1978, *55*, 718–727.
8) Smagula, Michael S.; Ho, Chi-Tang; Chang, Stephen S. The synthesis of 2-(2-pentenyl)furans and their relationship to the reversion flavor of soybean oil. *Journal of the American Oil Chemists' Society* 1979, *56*, 516–519.
9) Coleman, Edward C.; Ho, Chi-Tang. Chemistry of baked potato flavor. I. Pyrazines and thiazoles identified in the volatile flavor of baked potato. *Journal of Agricultural and Food Chemistry* 1980, *28*, 66–68.
10) Ho, Chi-Tang; Coleman, Edward C. Chemistry of baked potato flavor: Further identification of heterocyclic compounds in the volatile flavor of baked potato. *Journal of Food Science* 1980, *45*, 1094–1095.
11) Ouyang, J.-M.; Daun, Henryk; Chang, Stephen S.; Ho, Chi-Tang. Formation of carbonyl compounds from β–carotene during palm oil deodorization. *Journal of Food Science* 1980, *45*, 1214–1217 & 1222.
12) Coleman, Edward C.; Ho, Chi-Tang; Chang, Stephen S. Isolation and identification of volatile compounds from baked potatoes. *Journal of Agricultural and Food Chemistry* 1981, *29*, 42–48.
13) Ho, Chi-Tang; Coleman, Edward C. Halogen compounds identified in the volatile constituents of baked potatoes. *Journal of Agricultural and Food Chemistry* 1981, *29*, 200–201.
14) Chen, Ernest C.-H.; Ho, Chi-Tang. Identification of 9-decenoic acid in beer and yeast. *Journal of the American Society of Brewing Chemists* 1981, *39*, 70–71.
15) Lee, Min-Hsiung; Ho, Chi-Tang; Chang, Stephen S. Thiazoles, oxazoles and oxazolines identified in the volatile flavor of roasted peanuts. *Journal of Agricultural and Food Chemistry* 1981, *29*, 684–686.
16) Lee, Ken N.; Ho, Chi-Tang; Giorlando, Carl S.; Peterson, Robert J.; Chang, Stephen S. Methyl 3,4-dimethyl-5,6-dihydro-α–pyran-6-carboxylate in roasted beef volatiles: Identification and synthesis. *Journal of Agricultural and Food Chemistry* 1981, *29*, 834–836.
17) Ho, Chi-Tang; Tuorto, Raymond M. Mass spectra and sensory properties of some 4,5-dialkyloxazoles. *Journal of Agricultural and Food Chemistry* 1981, *29*, 1306–1308.
18) Nofal, Mostafa A.; Ho, Chi-Tang; Chang, Stephen S. New constituents identified in Egyptian jasmine absolute. *Perfumer & Flavorist* 1981, *6(6)*, 24–34.
19) Ho, Chi-Tang; Lee, Min-Hsiung; Chang, Stephen S. Isolation and identification of volatile compounds from roasted peanuts. *Journal of Food Science* 1982, *47*, 127–133.
20) Ho, Chi-Tang; Jin, Qi Zhang; Lee, Ken N.; Carlin, James T. 2-Acetyl-5-chloropyrrole in the volatile flavor constituents of cocoa butter. *Journal of Agricultural and Food Chemistry* 1982, *30*, 362–364.
21) Ho, Chi-Tang; Hartman, Guy J. Formation of oxazolines and oxazoles in Strecker degradation of DL-alanine and L-cysteine with 2,3-butanedione. *Journal of Agricultural and Food Chemistry* 1982, *30*, 793–794.
22) Ho, Chi-Tang; Jin, Qi Zhang; Lee, Ken N.; Carlin, James T. The identification of 2-acetyl-5-bromopyrrole in the volatiles of cocoa butter. *Lebensmittel-Wissenschaft und -Technologie* 1982, *15*, 169–170.
23) Wu, James W.; Lee, Min-Hsiung; Ho, Chi-Tang; Chang, Stephen S. Elucidation of the chemical structures of natural antioxidants isolated from rosemary. *Journal of the American Oil Chemists' Society* 1982, *59*, 339–345.
24) Nofal, Mostafa A.; Ho, Chi-Tang; Chang, Stephen S. Major volatile components of Egyptian rose absolute. *Perfumer & Flavorist* 1982, *7(4)*, 23–26.
25) Onyewu, Philip N.; Daun, Henryk; Ho, Chi-Tang. Formation of two thermal degradation products of β–carotene. *Journal of Agricultural and Food Chemistry* 1982, *30*, 1147–1151.
26) Hartman, Guy J.; Ho, Chi-Tang. Volatile compounds formed in the thermal degradation of hydrated collagen. *Lebensmittel-Wissenschaft und -Technologie* 1982, *15*, 253–254.

27) Hsu, Chen-Ming; Peterson, Robert J.; Jin, Qi Zhang; Ho, Chi-Tang; Chang, Stephen S. Characterization of new volatile compounds in the neutral fraction of roasted beef flavor. *Journal of Food Science* 1982, *47*, 2068–2069 & 2071.

28) Ho, Chi-Tang; Ichimura, Nobutomo. Identification of heterocyclic compounds in the volatile flavor of fresh tomato. *Lebensmittel-Wissenschaft und -Technologie* 1982, *15*, 340–342.

29) Ho, Chi-Tang; Jin, Qi Zhang. Synthesis and Identification of three 2-alkylbenzothiazoles in the volatile flavor constituents of roasted peanuts. *Lebensmittel-Wissenschaft und -Technologie* 1982, *15*, 366–367.

30) Ho, Chi-Tang; Hartman, Guy J.; Jin, Qi Zhang. The formation of oxazoles and oxazolines in the Strecker degradation of DL-methionine and L-cystine with 2,3-butanedione. *Lebensmittel-Wissenschaft und -Technologie* 1982, *15*, 368–371.

31) Ho, Chi-Tang; Jin, Qi Zhang. Mass spectra of some alkyloxazoles. *Journal of Agricultural and Food Chemistry* 1983, *31*, 180–181.

32) Chang, Stephen S.; Shen, Guo-Hui; Tang, Jian; Jin, Qi Zhang; Shi, Huang; Carlin, James T.; Ho, Chi-Tang. Isolation and identification of 2-pentenylfurans in the reversion flavor of soybean oil. *Journal of the American Oil Chemists' Society* 1983, *60*, 553–557.

33) Ho, Chi-Tang; Lee, Ken N.; Jin, Qi Zhang. Isolation and identification of volatile flavor compounds in fried bacon. *Journal of Agricultural and Food Chemistry* 1983, *31*, 336–342.

34) Nofal, Mostafa A.; Ho, Chi-Tang; Chang, Stephen S. Gas chromatographic characterization of jasmine absolute in relation to season. *Perfumer & Flavorist* 1983, *8(1)*, 75–80.

35) Hartman, Guy J.; Jin, Qi Zhang; Collins, George J.; Lee, Ken N.; Ho, Chi-Tang; Chang, Stephen S. Nitrogen-containing heterocyclic compounds identified in the volatile flavor constituents of roasted beef. *Journal of Agricultural and Food Chemistry* 1983, *31*, 1030–1031.

36) Ho, Chi-Tang; Jin, Qi Zhang; Lee, Ken N.; Carlin, James T.; Chang, Stephen S. Synthesis and aroma properties of new alkyloxazoles and alkylthiazoles identified in cocoa butter from roasted cocoa beans. *Journal of Food Science* 1983, *48*, 1570–1571.

37) Tang, Jian; Jin, Qi Zhang; Shen, Guo-Hui; Ho, Chi-Tang; Chang, Stephen S. Isolation and identification of volatile compounds in fried chicken. *Journal of Agricultural and Food Chemistry* 1983, *31*, 1287–1292.

38) Ho, Chi-Tang; Jin, Qi Zhang; Lee, Min-Hsiung; Chang, Stephen S. Positive identification of new alkyloxazoles, alkylthiazoles and piperidine in roasted peanut flavor. *Journal of Agricultural and Food Chemistry* 1983, *31*, 1384–1386.

39) Hartman, Guy J.; Scheide, Jürgen D.; Ho, Chi-Tang. Formation of volatile compounds from the reaction of leucine and D-glucose in propylene glycol. *Perfumer & Flavorist* 1983, *8(6)*, 81–86.

40) Hartman, Guy J.; Scheide, Jürgen D.; Ho, Chi-Tang. Effect of water activity on the major volatiles produced in a model system approximating cooked meat. *Journal of Food Science* 1984, *49*, 607–613.

41) Hartman, Guy J.; Ho, Chi-Tang. Volatile products of the reaction of sulfur-containing amino acids with 2,3-butanedione. *Lebensmittel-Wissenschaft und -Technologie* 1984, *17*, 171–174.

42) Hartman, Guy J.; Scheide, Jürgen D.; Ho, Chi-Tang. Volatile products formed from a flavor model system at high and low moisture levels. *Lebensmittel-Wissenschaft und -Technologie* 1984, *17*, 222–225.

43) Houlihan, Christopher M.; Ho, Chi-Tang; Chang, Stephen S. Elucidation of the chemical structure of a novel antioxidant, rosmaridiphenol, isolated from rosemary. *Journal of the American Oil Chemists' Society* 1984, *61*, 1036–1039.

44) Kim, Heasook; Ho, Chi-Tang; Chang, Stephen S. Isolation and identification of volatile flavor compounds in commercial oil-free soybean lecithin. *Journal of the American Oil Chemists' Society* 1984, *61*, 1235–1238.

45) Hartman, Guy J.; Carlin, James T,; Hwang, Shy-Shiuan; Bao, Yongde; Tang, Jian; Ho, Chi-Tang. Identification of 3,5-diisobutyl-1,2,4-trithiolane and 2-isobutyl-3,5-diisopropylpyridine in fried chicken flavor. *Journal of Food Science* 1984, *49*, 1398 & 1400.

46) Hartman, Guy J.; Carlin, James T.; Scheide, Jürgen D.; Ho, Chi-Tang. Volatile products formed from the thermal degradation of thiamin at high and low moisture levels. *Journal of Agricultural and Food Chemistry* 1984, *32*, 1015–1018.

47) Jin, Qi Zhang; Hartman, Guy J.; Ho, Chi-Tang. Aroma properties of some oxazoles. *Perfumer & Flavorist* 1984, *9(4)*, 25–29.

48) Ho, Chi-Tang; Jin, Qi Zhang. Aroma properties of some alkylthiazoles. *Perfumer & Flavorist* 1984, *9(6)*, 15–18.

49) Houlihan, Christopher M.; Ho, Chi-Tang; Chang, Stephen S. The structure of rosmariquinone, a new antioxidant isolated from *Rosmarinus officinalis* L. *Journal of the American Oil Chemists' Society* 1985, *62*, 96–98.

50) Shu, Chi-Kuen; Hagedorn, Myrna L.; Mookherjee, Braja D.; Ho, Chi-Tang. Volatile components of the thermal degradation of cystine in water. *Journal of Agricultural and Food Chemistry* 1985, *33*, 438–442.

51) Shu, Chi-Kuen; Hagedorn, Myrna L.; Mookherjee, Braja D.; Ho, Chi-Tang. pH effect on the volatile components in the thermal degradation of cysteine. *Journal of Agricultural and Food Chemistry* 1985, *33*, 442–446.

52) Shu, Chi-Kuen; Mookherjee, Braja D.; Ho, Chi-Tang. Volatile components of the thermal degradation of 2,5-dimethyl-4-hydroxy-3(2H)-furanone. *Journal of Agricultural and Food Chemistry* 1985, *33*, 446–448.

53) Shu, Chi-Kuen; Hagedorn, Myrna L.; Mookherjee, Braja D.; Ho, Chi-Tang. Two novel 2-hydroxy-3(2H)-thiophenones from the reaction between cystine and 2,5-dimethyl-4-hydroxy-3(2H)-furanone. *Journal of Agricultural and Food Chemistry* 1985, *33*, 638–641.

54) Raghavan, Sree K,; Ho, Chi-Tang; Daun, Henryk. Identification of soy proteins in meat by pyrolysis - high resolution gas chromatography. *Journal of Chromatography* 1986, *351*, 195–202.

55) Sotirhos, Nikolaos; Ho, Chi-Tang; Chang, Stephen S. High performance liquid chromatographic analysis of soybean phospholipids. *Fette Seifen Anstrichmittel* 1986, *88*, 6–8.

56) Sotirhos, Nikolaos; Ho, Chi-Tang; Chang, Stephen S. HPLC analysis of oxidative and polymerized decomposition products in commercial vegetable oils and heated fats. *Fette Seifen Anstrichmittel* 1986, *88*, 45–48.

57) Mensah, Tony A.; Ho, Chi-Tang; Chang, Stephen S. Products identified from photosensitized oxidation of selected furanoid flavor compounds. *Journal of Agricultural and Food Chemistry* 1986, *34*, 336–338.

58) Shu, Chi-Kuen; Hagedorn, Myrna L.; Ho, Chi-Tang. Two novel thiophenes identified from the reaction between cysteine and 2,5-dimethyl-4-hydroxy-3(2H)-furanone. *Journal of Agricultural and Food Chemistry* 1986, *34*, 344–346.

59) Chen, Chu-Chin; Ho, Chi-Tang. High performance liquid chromatographic determination of cyclic sulfurous compounds of Shiitake mushroom (*Lentinus edodes* Sing). *Journal of Chromatography* 1986, *356*, 455–459.

60) Chen, Chu-Chin; Rosen, Robert T.; Ho, Chi-Tang. Chromatographic analyses of gingerol compounds in ginger (*Zingiber officinale* Roscoe) extracted by liquid carbon dioxide. *Journal of Chromatography* 1986, *360*, 163–173.

61) Chen, Chu-Chin; Rosen, Robert T.; Ho, Chi-Tang. Chromatographic analyses of shogaol compounds derived from isolated gingerol compounds of ginger (*Zingiber officinale* Roscoe). *Journal of Chromatography* 1986, *360*, 175–184.

62) Chen, Chu-Chin; Kuo, May-Chien; Wu, Chung-May; Ho, Chi-Tang. Pungent compounds of ginger (*Zingiber officinale* Roscoe) extracted by liquid carbon dioxide. *Journal of Agricultural and Food Chemistry* 1986, *34*, 477–480.

63) Hwang, Shy-Shiuan; Carlin, James T.; Bao, Yongde; Hartman, Guy J.; Ho, Chi-Tang. Characterization of volatile compounds generated from the reactions of aldehydes with ammonium sulfide. *Journal of Agricultural and Food Chemistry* 1986, *34*, 538–542.

64) Carlin, James T.; Jin, Qi Zhang; Huang, Tzou-Chi; Ho, Chi-Tang; Chang, Stephen S. Identification of alkyloxazoles in the volatile compounds from french fried potatoes. *Journal of Agricultural and Food Chemistry* 1986, *34*, 621–623.

65) Carlin, James T.; Lee, Ken N.; Hsieh, Oliver A.-L.; Hwang, Lucy S.; Ho, Chi-Tang; Chang, Stephen S. Comparison of acidic and basic volatile compounds of cocoa butter from roasted and unroasted cocoa beans. *Journal of the American Oil Chemists' Society* 1986, *63*, 1031–1036.

66) Chen, Chu-Chin; Kuo, May-Chien; Ho, Chi-Tang. High performance liquid chromatographic determination of pungent gingerol compounds of ginger (*Zingiber officinale* Roscoe). *Journal of Food Science* 1986, *51*, 1364–1365.

67) Chen, Chu-Chin; Ho, Chi-Tang. Identification of sulfurous compounds of Shiitake mushroom (*Lentinus ododes* Sing). *Journal of Agricultural and Food Chemistry* 1986, *34*, 830–833.

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# Differential Effects of Theaflavin Monogallates on Cell Growth, Apoptosis, and *Cox-2* Gene Expression in Cancerous *versus* Normal Cells[1]

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## ABSTRACT

**Theaflavin (TF-1), theaflavin-3-monogallate and theaflavin-3′-monogallate mixture (TF-2), and theaflavin-3,3′-digallate (TF-3) are the major black tea polyphenols. Here we compared the effects of these polyphenols on cell growth, apoptosis, and gene expression in normal and cancerous cells. We showed that TF-2 (10–50 μM) inhibited the growth of SV40 transformed WI38 human cells (WI38VA) and Caco-2 colon cancer cells but had little effect on the growth of their normal counterparts. The $IC_{50}$s of TF-2 for the growth inhibition of WI38 and WI38VA cells were, respectively, 300 and 3 μM. The other two black tea polyphenols, TF-1 and TF-3, did not exhibit such differential growth-inhibitory effect. TF-2, but not TF-1 or TF-3, induced apoptosis in transformed WI38VA cells but not in normal WI38 cells, suggesting that apoptosis was responsible, at least in part, for the differential growth-inhibitory effect of TF-2. Cox-2 has been implicated in intestinal carcinogenesis. Among the tea polyphenols tested, TF-2 and, to a lesser degree, (−)-epigallocatechin gallate inhibited *cyclooxygenase (Cox)-2* gene expression. TF-2 at 50 μM completely blocked the serum-induced *Cox-2* gene expression at both mRNA and protein level. Other genes, including c-*fos*, c-*myc*, *thymidine kinase*, *proliferating cell nuclear antigen*, *BRCA1*, *BRCA2*, and *Cox-1*, were not significantly affected by TF-2. These findings suggest that TF-2 may be responsible, at least in part, for the chemopreventive activity in black tea extracts.**

## INTRODUCTION

Epidemiological studies suggest that tea may have a protective role against certain human cancers (reviewed in Refs. 1–3). Catechin polyphenols in green tea have been shown to inhibit the proliferation of cultured mammalian cells including colon carcinoma, lung carcinoma, breast carcinoma, melanoma, and leukemic cells (4, 5). We have reported that EGCG, a major green tea catechin polyphenol, inhibits the growth of human tumor cells, including Caco-2 colorectal cancer cells, Hs578T breast cancer cells, and SV40-transformed WI38 cells but has little or no inhibitory effect on the growth of their normal counterparts (6). Black tea extract has been shown to be potent in inhibiting tumorigenesis in animal model systems, including skin (7), lung (8), colon (9, 10), esophagus (11), and mammary gland (10, 12). The major black tea polyphenols, TF-1, TF-2, and TF-3, are biochemical oxidation products derived from green tea polyphenols and are responsible for the characteristic color, fragrance, and taste of black tea (1–3). TF-3 has been shown to be as potent as EGCG in inhibiting the growth of human A431 carcinoma cells (13). The biological effects of each individual black tea polyphenol have not been compared or studied in detail at the molecular level. In this study, we compared the effects of TF-1, TF-2, and TF-3 on cell proliferation, apoptosis, and gene expression in cancerous human cells (WI38VA and Caco-2 colon cancer cells) and in their normal counterparts (WI38 diploid fibroblasts, CCD-33Co, and FHC colorectal cells). We found that, among the three black tea polyphenols tested, only TF-2 exhibited a clear differential growth-inhibitory and apoptotic effect toward cancerous cells.

The two isoforms of cyclooxygenase, constitutive Cox-1 and inducible Cox-2, are key enzymes for prostaglandin biosynthesis (reviewed in Ref. 14). An elevation of the Cox-2 activity has been associated with certain pathological processes, including colon cancer (14–17). A direct link between Cox-2 expression level and polyps formation has been demonstrated in APC knockout mice, suggesting that Cox-2 plays an important role in colon cancer formation (15). In light of the important role of the *Cox-2* gene in intestinal carcinogenesis and other inflammatory processes (14–17), we have also examined whether any of the tea polyphenol may affect the expression of Cox-2 and other growth-related genes in colon cancer cells. We found that TF-2 specifically inhibited *Cox-2* gene expression at both the mRNA and protein level.

## Materials and Methods

**Materials.** DMEM and fetal bovine serum were obtained from Life Technologies, Inc. (Gaithersburg, MD). Other chemicals were from Sigma Chemical Co. (St. Louis, MO). [α-$^{32}$P]dATP (>3000 Ci/mmol) was purchased from ICN Chemical (Radioisotope Division, Irvine, CA). Theaflavin polyphenols were isolated and purified from black tea powder as described previously (18). The structures of these compounds are shown in Fig. 1. The TF-2 used in this study contains two theaflavin monogallate isomers.

**Tissue Culture.** Normal human WI38 (cell strain AG06814E; PDL=16) and the SV40 virally transformed WI38 cells (cell strain AG07217) were obtained from Coriell Institute for Medical Research (Camden, NJ). Human colon cancer cell, Caco-2 (ATCC HTB-37), normal human colon cells CCD-33Co (ATCC CRL-1539), and FHC (ATCC CRL-1831) were purchased from American Type Culture Collection (Rockville, MD). Cells were cultured in Dulbecco's medium containing 10% fetal bovine serum at 37°C, 5% $CO_2$. For proliferation assay, cells were plated at about $2 \times 10^5$ cells per 35-mm dish, with or without tea polyphenol, and the number of viable cells, as determined by trypan blue dye exclusion, was counted under a phase contrast microscope. To alleviate the concern of the degradation of theaflavins during incubation, we also replenished the culture with fresh growth medium containing the tea chemical once every other day. We did not find any significant difference in the results obtained by either method. Proliferation of human fibroblasts was also estimated by a crystal violet staining method. Cells were seeded in a standard 24-well tissue culture plate at about $1 \times 10^5$ cells/ml in the presence of black tea polyphenol. On the 4th or 5th day after plating, cells were fixed with 5% trichloroacetic acid and stained with Bacto Gram Crystal Violet solution (Difco, Detroit, MI). The staining intensity of the fibroblast culture correlated well with the number of viable cells in the culture as determined by cell counting (data not shown).

**TUNEL Assay.** Apoptosis was analyzed by TUNEL assay as described previously (6). Briefly, cultures at ~90% confluency were treated with TF-2 (100 μM) for 18 h. Cells were fixed with 4% of formaldehyde solution, washed, and incubated in a buffer containing fluorescein-12-dUTP and terminal deoxynucleotidyl transferase for 1 h. The nuclei of apoptotic cells exhibited green fluorescence using an FITC filter under fluorescent microscope.

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[3] The abbreviations used are: EGCG, (−)-epigallocatechin gallate; COX, cyclooxygenase; TF-1, theaflavin; TF-2, theaflavin-3-monogallate and theaflavin-3′-monogallate mixture; TF-3, theaflavin-3,3′-digallate; ATCC, American Type Culture Collection; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TK, thymidine kinase; PCNA, proliferating cell nuclear antigen.

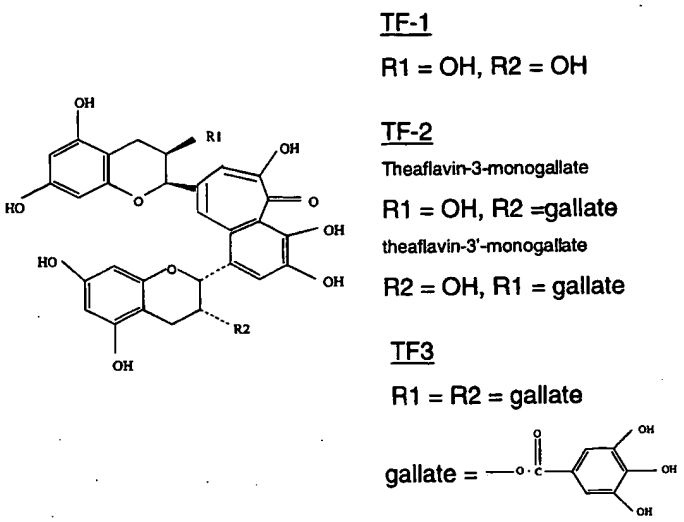


Fig. 1. Chemical structures of TF-1, TF-2, and TF-3.

**DNA Fragmentation Analysis.** Confluent cultures were treated with tea chemicals at different concentrations for various times. Cells were harvested and suspended in a lysis buffer [10 mM Tris-HCl (pH 8.0), 100 mM NaCl, 25 mM EDTA, 0.5% SDS, and 100 μg/ml proteinase K] for 20 h at 37°C. DNA was extracted with a phenol-chloroform mixture, precipitated by ethanol, dried, and dissolved in a TE buffer. RNA was digested with 2 μg/ml of RNase Cocktail (Ambion, Austin, Texas). The DNA samples were analyzed by electrophoresis on a 1% agarose gel containing ethidium bromide (0.5 μg/ml).

**Northern Blot Analysis.** Total RNA samples were resolved by electrophoresis on 1% agarose-formaldehyde gel (6 μg/lane) and transferred onto a nylon membrane. Northern blot analysis was performed as described previously (19).

**RT-PCR.** Serum, growth factors, cytokines, or phorbol esters can induce *Cox-2* gene expression (20, 21). In this study, fresh serum was used to induce *Cox-2* and other growth-related genes in quiescent cultures. Confluent cultures were serum-deprived for 48 h to become quiescent and then stimulated with 10% fresh fetal bovine serum as described previously (6). Tea chemicals at various concentrations were added to the culture immediately after serum stimulation. No significant morphological change of Caco-2 cells was observed, indicating that Caco-2 cells did not undergo differentiation during serum deprivation (data not shown). Cells were harvested at indicated times for total RNA preparation using RNeasy Total RNA kit (Qiagen, Chatsworth, CA). Total RNA (1 μg) was reverse transcribed by incubating with SuperScript RNase H reverse transcriptase (Life Technologies, Inc., Grand Island, NY) using Oligo(dT)$_{12-18}$ as primer. For PCR amplification, gene-specific primers used are listed below: GAPDH sense, 5'-TGAAGGTCGGAGTCAACGGATTTGGT-3'; GAPDH antisense, 5'-CATGTGGGCCATGAGGTCCACCAC-3'; BRCA1 sense, 5'-CTCTGGGAAAGTATCGCTGTCATG-3'; BRCA1 antisense, 5'-AGAGGCATCCAGAAAAGTATCAGG-3'; BRCA2 sense, 5'-TGCTGCCAGTAGAAATTCTC-3'; BRCA2 antisense, 5'-CTTTGTCCAAAGATTCCTTTG-3'; ODC sense, 5'-AATCAACCCAGCGTTGGACAA-3'; ODC antisense, 5'-ACATCACATAGTAGATCGTCG-3'; TK sense, 5'-AGCACAGAGTTGATGAGACGC-3'; TK antisense, 5'-GCTTCCTCTGGAAGGTCCCAT-3'; PCNA sense, 5'-ACGTCTCTTTGGTGCAGCTC; PCNA antisense, 5'-CAAGTTGTTCAACATCTAAATCCATC-3'; COX1 sense, 5'-GTTCAACACCTCCATGTTGGTGGAC-3'; COX1 antisense, 5'-TGGTGTTGAGGCAGACCAGCTTC-3'; COX-2 sense, 5'-TTCAAATGAGATTGTGGGAAAAT-3'; COX-2 antisense, 5'-AGATCATCTCTGCCTGAGTATCTT-3'; c-myc sense, 5'-CAGGATCCGTGCATCGACCCCTCGGTG-3'; c-myc antisense, 5'-CGCCTAAGCTTTGACATTCTCCTCGGTG-3'; c-jun sense, 5'-CCAAGATCCTGAAACAGAGCATG-3'; c-jun antisense, 5'-TCCGAGTTCTGAGCTTTCAAGGT-3'; c-fos sense, 5'-ATGATGTTCTCGGGCTTCAACGCAG-3'; and c-fos antisense, 5'- CCGAAGAAGCCAGGCTCTAGTTAGCG-3'.

PCR was performed under conditions that allowed the amounts of PCR products to be proportional to the amounts of input RNA. GAPDH was used as an internal control. The PCR products were analyzed by electrophoresis on 1% agarose gel containing 0.5 μg/ml ethidium bromide.

**Western Blot Analysis.** Cells after various treatment were harvested in a lysis buffer [150 mM NaCl, 100 mM Tris (pH 8.0), 1% Tween 20, 1 mM EDTA, 50 mM DDT, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin]. The cell lysates were sonicated and centrifuged at 11,000 × *g* for 10 min. The supernatant containing 30 μg of protein was analyzed on a 10% SDS-PAGE under reducing conditions. The gel was transferred onto a nitrocellulose membrane, and the membrane was probed with anti-Cox-2 antibody (Cayman Chemical, Ann Arbor, MI) at 1:1000 dilution. The affinity purified goat antirabbit IgG conjugated to horseradish peroxidase (Bio-Rad Laboratories, Hercules, CA) was used as secondary antibody. The hybridized protein bands were detected using the ECL kit (Amersham Pharmacia, Piscataway, NJ).

## RESULTS

**Differential Inhibitory Effect of TF-2 on the Growth of WI38 and WI38VA Cells.** WI38 human diploid fibroblasts have a finite life span (22), whereas WI38VA, the SV40 virally transformed WI38 cells, are immortal and cancerous in nude mice (23). The dose-response effects of three black tea polyphenols on the growth of these two cell types were qualitatively compared by a crystal violet staining method. As shown in Fig. 2, only TF-2 exhibited a differential growth-inhibitory effect. Thus, TF-2 at 10 μM prominently inhibited the proliferation of WI38VA cells but had little or no inhibitory effect on the growth of normal WI38 cells. TF-1 at 50 μM inhibited the growth of both WI38 and WI38VA cells to the same extent, and TF-3 at 50 μM inhibited the growth of WI38 cells but not the growth of WI38VA cells. We then examined the effect of TF-2 on the growth kinetics and the morphology of WI38 and WI38VA cells. Fig. 3*A* shows clearly that TF-2 affected the growth rate of WI38 and WI38VA cells differently. Although the growth of WI38 cells was not significantly affected by TF-2 at 50 μM, TF-2 at 10 μM completely blocked the growth of WI38VA cells, consistent with the crystal violet staining data as shown in Fig. 2. The $IC_{50}$ of TF-2 for the growth inhibition was estimated to be 300 μM for WI38 and 3 μM for WI38VA cells, a difference of two orders of magnitude. Fig. 3*B* shows that although TF-2 did not affect the growth and viability of WI38 cells, it did cause a slight morphological change in the treated

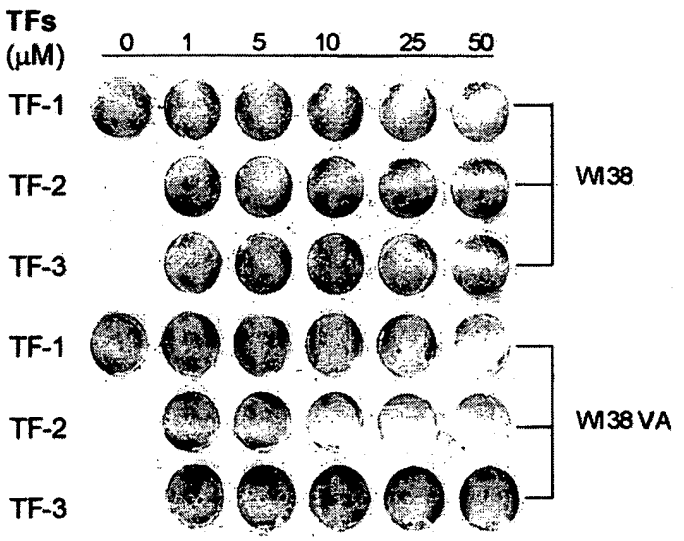


Fig. 2. Comparison of the effect of black tea polyphenols, TF-1, TF-2, and TF-3, on the proliferation of WI38 and WI38VA cells. Cells were seeded at 1 × $10^5$ cells/ml in a standard 24-well tissue culture plate and then treated with 0, 1, 5, 10, 25, and 50 μM TF-1, TF-2, or TF-3. Cells were fixed with trichloroacetic acid and stained with crystal violet 4 days after plating.

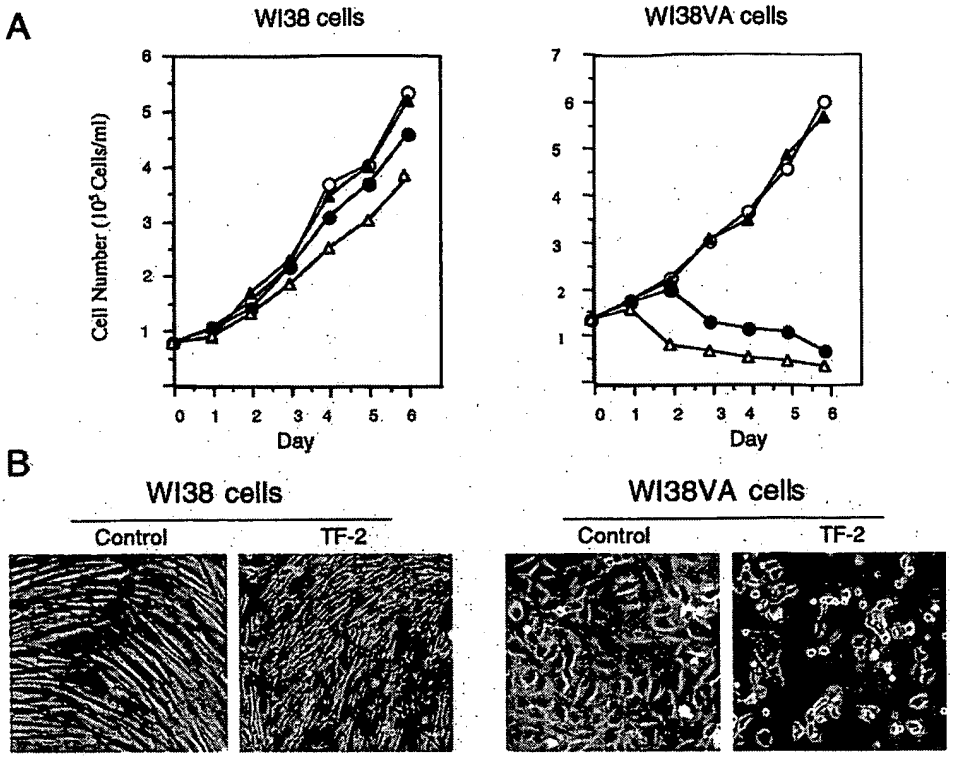


Fig. 3. *A*, effect of TF-2 on the growth rate of normal WI38 and SV40 transformed WI38VA cells. Cells were plated in a 35-mm dish on day 1 in the absence (○) or presence of 1 μM (▲), 10 μM (●), and 50 μM (△) of TF-2. The viable cells were counted at the indicated times. Each *point* represents an average of three separate dishes. *B*, WI38 and WI38VA cells were cultured in the absence (control) or presence 10 μM TF-2. Phase contrast micrographs from representative fields were taken 5 days after the treatment.

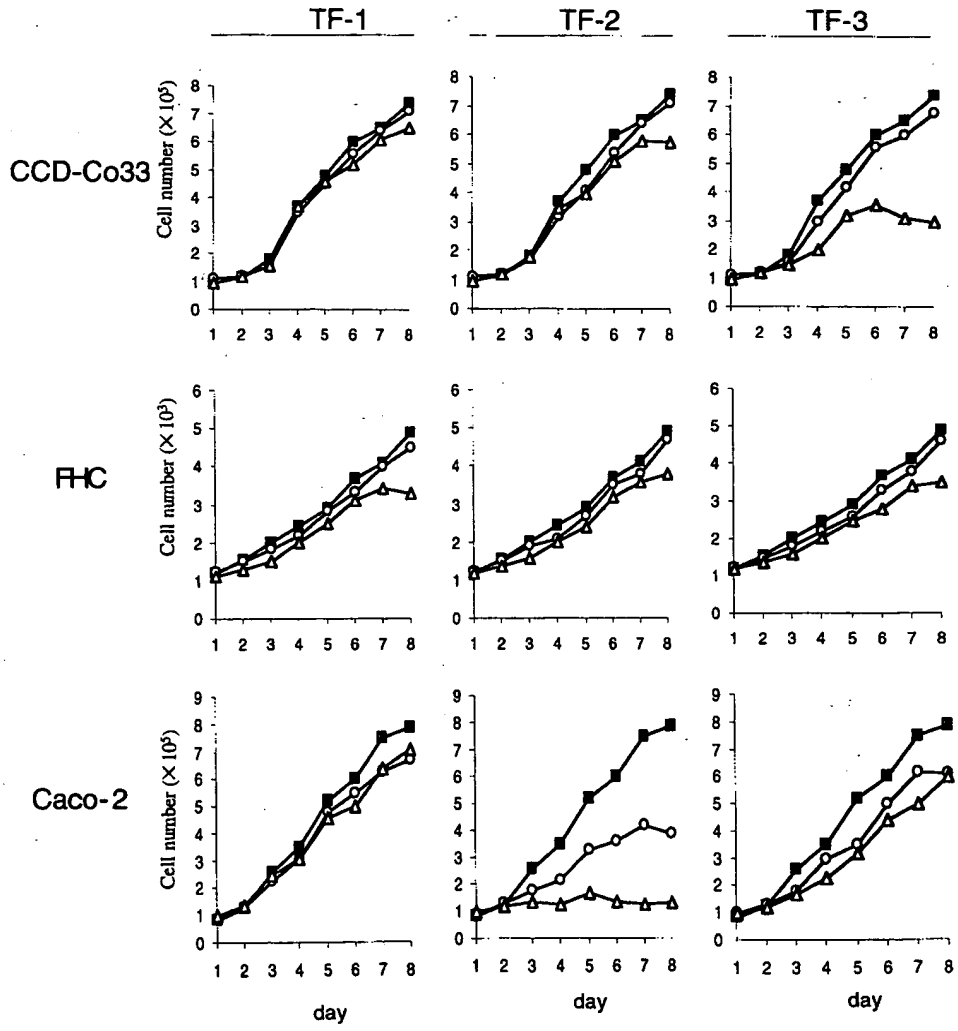


Fig. 4. Effect of three black tea polyphenols, TF-1, TF-2, and TF-3, on the growth kinetics of CCD-33Co colon cells, FHC colon cells, and Caco-2 colon cancer cells. Cells were plated in a 35-mm dish on day 1 in the absence (■) or presence of 10 μM (○) and 50 μM (△) of TF-1, TF-2, or TF-3. The viable cells were counted at indicated times. Each *point* represents an average of two separate dishes.

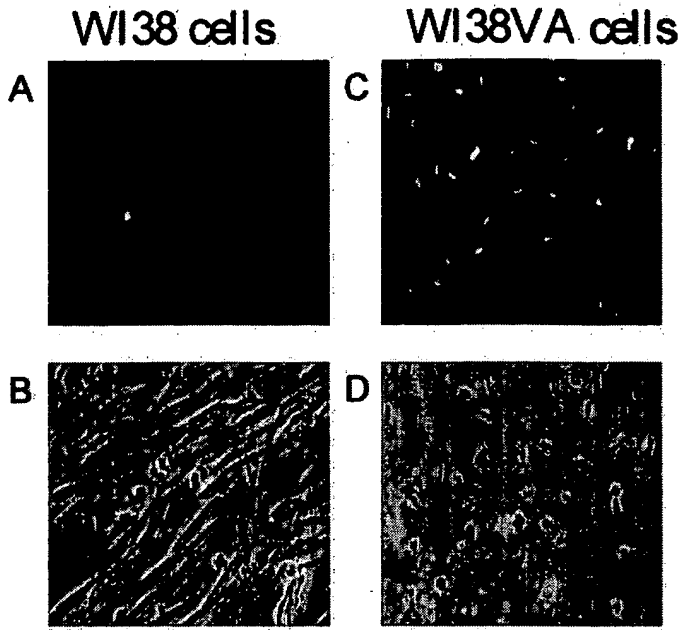


Fig. 5. TUNEL assay of the effect of TF-2 on apoptosis. WI38 and WI38VA cells at 90% confluency were treated with TF-2 at 100 μM for 18 h. Apoptotic cells were detected by labeling with fluorescein-12-dUTP using terminal deoxynucleotidyl transferase. The labeled cells were detected by a fluorescence microscope using an FITC filter (*A* and *C*). All cells, including apoptotic ones, in the cultures were also stained by propidium iodide and detected by a fluorescence microscope using a rhodamine filter (*B* and *D*).

cells (Fig. 3*B*, *left panels*). In the WI38VA cultures treated with TF-2, very few viable cells could be detected (Fig. 3*B*, *right panels*).

**Effect of Black Tea Theaflavins on the Growth of Normal and Cancerous Colon Cells.** Caco-2 colon cancer cells and CCD-33Co colorectal cells have been used as a normal and cancerous pair to study the biological effects of EGCG (6). Because CCD-33Co cells appear to be fibroblastic (data not shown), we also included the FHC normal human colon cell strain (epithelial origin; Ref. 24) in the present study. Fig. 4 shows the effects of the three black tea theaflavins on the growth of normal (CCD-33Co and FHC) and cancerous colorectal cells. TF-2 at 50 μM inhibited the growth of Caco-2 but had little effect on the growth of CCD-33Co or FHC cells. TF-1 and TF-3 did not exhibit such a differential growth-inhibitory effect.

**Differential Effect of TF-2 on the Induction of Apoptosis.** Because apoptosis could be a major cause for growth inhibition, we examined whether TF-2 may induce apoptosis differently in normal and transformed cells. We first used the TUNEL assay to examine this possibility. Fig. 5 shows that TF-2 caused almost every cell in the WI38VA culture to become apoptotic, as indicated by the green fluorescence attributable to fluorescein-12-dUTP labeling (Fig. 5, *C versus D*). In contrast, almost no cells in the normal WI38 culture exhibited green fluorescence after TF-2 treatment (Fig. 5, *A versus B*). We next compared the effects of the three black tea polyphenols on apoptosis using DNA fragmentation analysis. Fig. 6 shows that both TF-1 and TF-3 did not induce any appreciable DNA fragmentation in either WI38 or WI38VA cells (Fig. 6, *A* and *C*). In contrast, TF-2 caused an extensive DNA fragmentation in transformed WI38VA cells but not in WI38 cells (Fig. 6*B*). The propensity of transformed WI38VA for undergoing apoptosis in the presence of TF-2 could explain, at least in part, why TF-2 preferentially inhibited the growth of transformed cells. Because TF-1 and TF-3 have been reported to be capable of inducing apoptosis in human lymphoid leukemia cells and stomach tumor cells (25), the efficacy of tea polyphenol on apoptosis may be cell type dependent.

**Effect of TF-2 on *Cox-2* Gene Expression.** In light of the potential role of the *Cox-2* gene in colon cancer carcinogenesis, we have examined the effect of TF-2 and other tea polyphenols on *Cox-2* gene expression. Fig. 7*A* shows that TF-2 at 50–100 μM prominently suppressed the *Cox-2* gene expression in Caco-2 cells. TF-1 and TF-3 did not appear to have any significant effect on *Cox-2* gene expression. EGCG, a green tea polyphenol, was less potent than TF-2 in suppressing *Cox-2* gene expression.

We next examined the effect of TF-2 on the time course of *Cox-2* gene expression. Fig. 7*B* shows that the *Cox-2* mRNA was detectable in quiescent Caco-2 cells, consistent with the notion that colon cancer cells have elevated *Cox-2* gene expression (Fig. 7*B*, *Lane 1*). TF-2 not

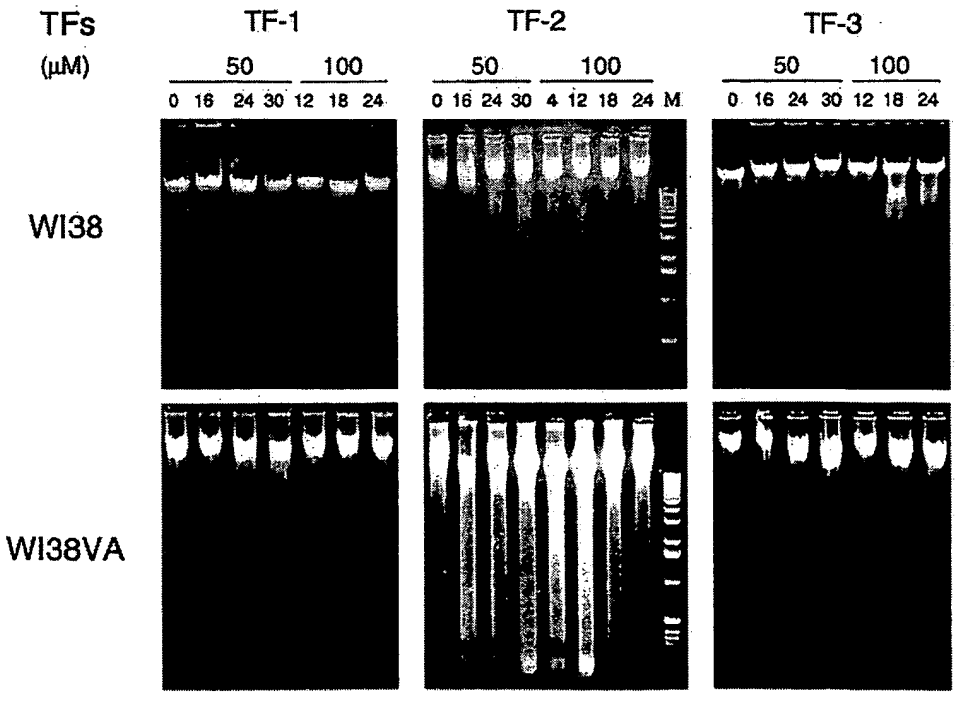


Fig. 6. DNA fragmentation analysis. WI38 and WI38VA cells at were treated with TF-1 (*A*), TF-2 (*B*), and TF-3 (*C*) at 50 or 100 μM. The cells were harvested at the indicated times, and the formation of a DNA internucleosomal ladder was monitored by agarose gel electrophoresis. *M*, DNA size markers.

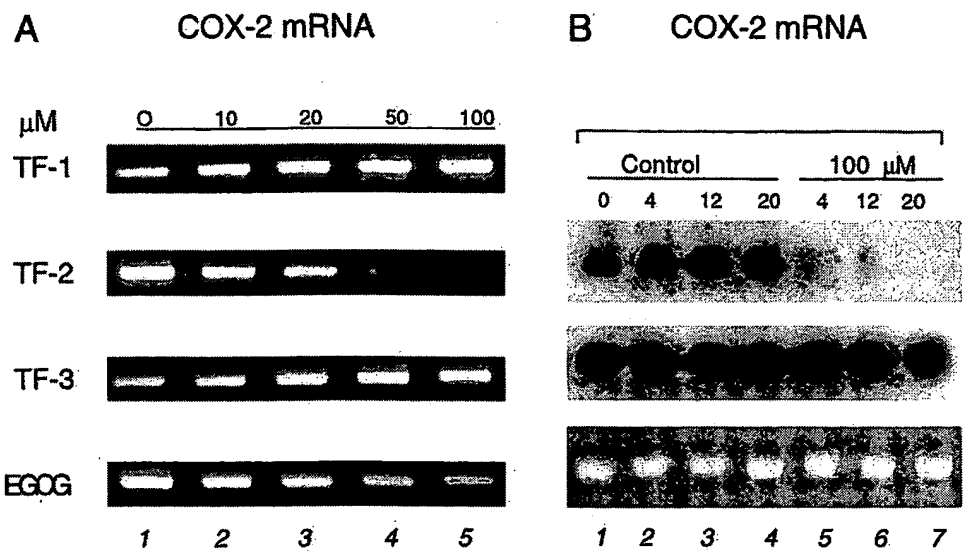


Fig. 7. *A*, effects of theaflavin chemicals and EGCG on the expression of the *Cox-2* gene in Caco-2 cells. Confluent cultures of Caco-2 cells were serum deprived for 48 h and then stimulated with 10% of fetal bovine serum in the presence of various tea polyphenol at indicated concentration for 4 h. *Cox-2* gene expression was determined by RT-PCR as described in "Materials and Methods." *B*, time course of *Cox-2* gene expression in Caco-2 cells. Cells at 90% confluency were serum-deprived for 36 h and then replenished with complete growth medium containing 10% fetal bovine serum, without (control) or with TF-2 at 100 μM. The cells were harvested at the indicated times for total RNA preparation. RNA samples were analyzed by Northern blot analysis as described in "Materials and Methods." GAPDH and 28S rRNA were used as internal standards.

only blocked the serum-induced increase in *Cox-2* gene expression but also abolished the basal level of *Cox-2* mRNA (Fig. 7*B*, *Lanes 5–7 versus Lanes 2–4*). Unlike Caco-2 cells, *Cox-2* mRNA was not detectable in quiescent WI38 or WI38VA cells (Fig. 8*A*, *Lanes 1* and *8*). The addition of fresh serum to these two fibroblastic cultures prominently induced the appearance of a 4.5-kb *Cox-2* transcript (Fig. 8*A*, *Lane 2 versus Lane 1* and *Lane 9 versus Lane 8*). However, the levels of induced *Cox-2* mRNA in WI38VA cells were much higher and more sustained than that in WI38 cells, suggesting that *Cox-2* mRNA may be more stable in transformed WI38VA cells (Fig. 8*A*, *Lane 10 versus Lane 3*). Again, TF-2 blocked the serum-induced increase in *Cox-2* gene expression in both WI38 and WI38VA cells (Fig. 8*A*, *Lanes 4–7* and *Lanes 11–14*). Consistent with the notion that *Cox-2* is transcriptionally regulated, Fig. 8*B* shows that TF-2 at 40 μM reduced the Cox-2 protein level in WI38 cells by ~50% (Fig. 8*B*, *Lane 4 versus Lane 1*) and completely eliminated Cox-2 protein in WI38VA cells (Fig. 8*B*, *Lane 9 versus Lane 6*).

**Effect of TF-2 on the Expression of Growth-related Genes.** To determine whether the effect of TF-2 on *Cox-2* gene expression could be a part of global suppression of serum-inducible genes, we examined the effect of TF-2 on the expression of other important genes. We included in this study growth-related genes such as c-*fos*, c-*myc*, *TK*, and PCNA. We also included *Cox-1* and the breast cancer-related tumor suppressor genes, *BRCA1* and *BRCA2*. Fig. 9 shows that among all of these genes, the only one that was dramatically attenuated by TF-2 was *Cox-2*. The constitutive *Cox-1* gene was completely insen-

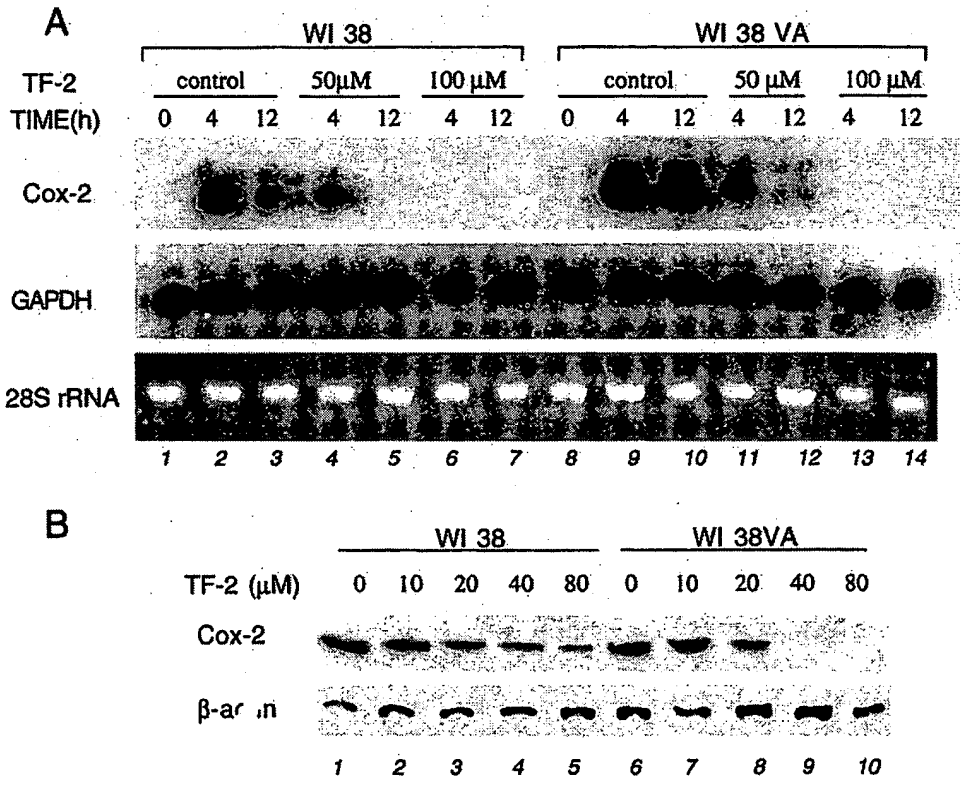


Fig. 8. Effects of TF-2 on *Cox-2* gene expression in WI38 and WI38VA cells. *A*, Northern blot analysis. Cells at 90% confluency were serum-deprived for 48 h and then replenished with complete growth medium containing 10% fetal bovine serum without (control) or with TF-2 at various concentrations. The cells were harvested at the indicated time for total RNA preparation. RNA samples were used for Northern blot analysis as described in "Materials and Methods." The levels of GAPDH mRNA and 28S rRNA were used as internal standards. *B*, Western blot analysis. Confluent cultures of WI38 and WI38VA cells were serum-deprived for 48 h and then serum stimulated with 10% fetal bovine serum for 8 h in the presence of TF-2 at the indicated concentrations. Cells were harvested, and whole-cell extracts were prepared for Western blot analysis using anti-Cox-2 antibody and anti-actin antibody as described in "Materials and Methods." Each lane contained 30 μg of proteins. The actin was used as an internal standard.

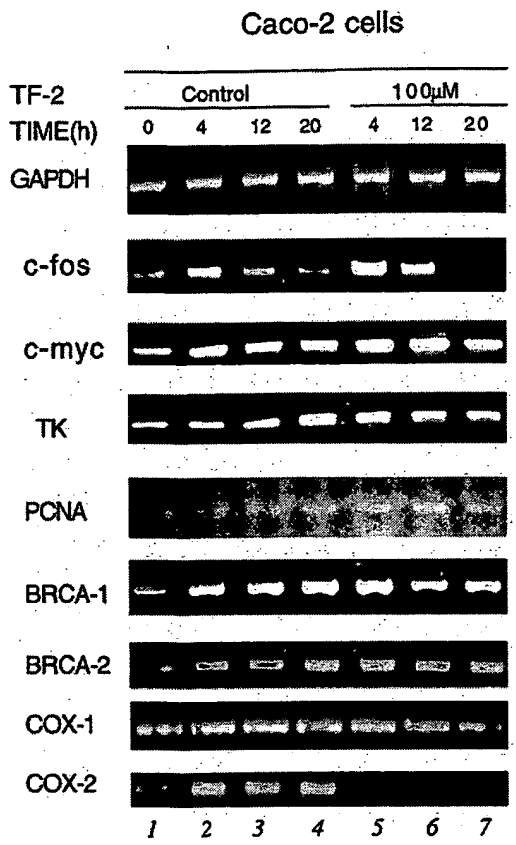


Fig. 9. Effect of TF-2 on the expression of growth-related genes. Caco-2 cells at 90% confluency were serum-deprived for 48 h and then stimulated with 10% fetal bovine serum in the absence (*Lanes 1–4, Control*) or presence of TF-2 (*Lanes 5–7, 100 μM*). The cells were harvested at the indicated time for total RNA preparation. The relative level of mRNA of each gene was analyzed by RT-PCR as described in "Materials and Methods." The mRNA level of housekeeping gene, *GAPDH,* was used as an internal standard.

sitive to TF-2, indicating that the action of TF-2 on the suppression of *Cox-2* gene expression is highly specific.

## DISCUSSION

Epidemiological studies suggest that tea may reduce cancer risk (1–3). In addition, chemopreventive effects of tea extracts have been demonstrated in animal models for cancers of the skin, lung, esophagus, mammary glands, and colon (1–11). To understand the molecular basis underlying the biological effects of tea extracts, we have investigated the effects of three black tea polyphenols on growth, apoptosis, and gene expression in normal and cancerous human cells.

Among the three black tea polyphenols tested, TF-2 exhibited a striking differential growth-inhibitory effect for at least two cancerous cell lines, WI38VA and Caco-2 (Figs. 2–4). The fact that TF-2 was potent in inducing apoptosis in WI38VA cells but not in WI38 cells (Figs. 5 and 6) suggests that apoptosis may contribute to the differential growth-inhibitory effect of TF-2. Because TF-1 and TF-3 did not induce apoptosis in either WI38 or WI38VA cells (Fig. 6, *A* and *C*), we suspect that TF-2 may target specifically some components involved in apoptotic pathways in cancerous cells.

Inhibition of Cox enzyme by nonsteroidal anti-inflammatory drugs can reduce the risk of colon cancer (26, 27). Specific inhibition of *Cox-2* gene expression could be used as an alternative means for treating inflammation and diseases that are associated with *Cox-2* elevation (28, 29). The finding that TF-2 inhibited *Cox-2* gene expression is interesting in several regards: (*a*) other black tea theaflavins, TF-1 and TF-3 at 100 μM, did not inhibit *Cox-2* gene expression (Fig. 7); (*b*) the $IC_{50}$ of TF-2 in inhibiting *Cox-2* gene expression was about 20–40 μM, comparable with that of nonsteroidal anti-inflammatory drugs (Figs. 7 and 8); and (*c*) TF-2 did not inhibit the expression of the constitutive *Cox-1* gene and other growth-related genes including c-*fos,* c-*myc, TK,* and *PCNA* (Fig. 9). Nonetheless, much work still needs to be done to assess the potential therapeutic promise of TF-2 *in vivo*.

Tea polyphenols generally exhibit antioxidative effects (30, 31), inhibit the AP-1 binding activity (32), and block the autophosphorylation of the epidermal growth factor and platelet-derived growth factor receptors (13). However, these biological effects are unlikely to be involved in *Cox-2* gene regulation, because neither TF-1 nor TF-3 shared the inhibitory action of TF-2 on *Cox-2* gene expression (Fig. 7*A*). Because the *Cox-2* gene is controlled primarily at the transcription level (33, 34), TF-2 may specifically affect the binding of certain *trans*-acting factors such as CCAAT/enhancer-binding proteins or nuclear factor-κB to the Cox-2 promoter. This possibility is currently under investigation.

In summary, we showed that, among the black tea polyphenols, TF-2 was unique in that it was a potent inhibitor of cancer cell growth, it differentially induced apoptosis in transformed cells, and it specifically inhibited *Cox-2* gene expression. These features make TF-2 a useful tea compound for further evaluation as a potential therapeutic reagent.

## ACKNOWLEDGMENTS

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# Herbs: Challenges in Chemistry and Biology

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# Foreword

The ACS Symposium Series was first published in 1974 to pr vide a mechanism for publishing symposia quickly in book form. Tl purpose of the series is to publish timely, comprehensive books dev oped from ACS sponsored symposia based on current scientific r search. Occasionally, books are developed from symposia sponsored l other organizations when the topic is of keen interest to the chemist audience.

Before agreeing to publish a book, the proposed table of co tents is reviewed for appropriate and comprehensive coverage and f interest to the audience. Some papers may be excluded to better foc the book; others may be added to provide comprehensiveness. Whe appropriate, overview or introductory chapters are added. Drafts chapters are peer-reviewed prior to final acceptance or rejection, ar manuscripts are prepared in camera-ready format.

As a rule, only original research papers and original revie papers are included in the volumes. Verbatim reproductions of prev ously published papers are not accepted.

**ACS Books Department**

# Contents

## Natural Product Chemistry and Analysis







## Biological Activities

# Preface

There is an increasing use of botanicals, botanical extracts, and purified natural compounds for the treatment and prevention of disease, especially chronic disease, in North America and Europe—not to mention Asian countries, which already have a long history of applying herbal medicines. For example, in China, herbal medicine (Traditional Chinese Medicine) is viewed as a national treasure, and has equal status with Western medicine. Traditional Chinese medicine is well-known to be effective for all kinds of disease, especially for age-related diseases.

In the United States, research and product development in nutraceuticals and functional foods are producing many novel botanicals, vitamins, and/or mineral products that are appearing in the market. The reevaluation of botanical products is, however, accompanied by higher expectations from nutritionalists, physicians, pharmacists, and consumers who demand quality, safety, and efficacy from these products. With the continuing reports of the side effects and toxicity of popular botanical products such as Ephedra and Kava, consumers are starting to back away from botanical products and are beginning to lose faith in dietary supplements. Thus, it is time to present updated research relating to the quality control, chemical, and pharmacological respects of herbal products in order to regain the confidence of the public.

The symposium upon which this book is based was organized to bring together leading researchers from the United States, China, Taiwan, Japan, and Canada to discuss future research directions in herbal products and to share works in botanical research. The symposium was held in Anaheim, California from March 28 to April 1, 2004 as part of the 227$^{th}$ American Chemical Society National meeting.

This book is divided into four sections. In the overview (first) section, Dr. Betz first describes the National Institutes of Health's vision of clearing confusion in the marketplace via validated analytical methods and referenced botanical materials. This is followed by Chapter 2 on the development of patentable new drugs from Traditional Chinese Medicine, Chapter 3 about bioactive polyphenols from foods and dietary supplements, Chapter 4 about the application of instrumental methods to the analysis and the prevention of adulteration in popular dietary supplements in the market, and Chapter 5 about assessing bioactive botanical

Chapter 24

# Effect of Black Tea Theaflavins on 12-*O*-Tetradecanoylphorbol-13-acetate-Induced Inflammation

## Expression of Pro-Inflammatory Cytokines and Arachidonic Acid Metabolism in Mouse Ear and Colon Carcinogenesis in Min (Apc+/–) Mice

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We have examined the effects of black tea constituents, theaflavin mixture (TFs), on 12-*O*-tetradecanoylphorbol-13-acetate-induced inflammation, up-expression of pro-inflammatory cytokines, interleukin-1β (IL-1β) and interleukin-6 (IL-6) protein levels and formation of prostaglandin $E_2$ ($PGE_2$) and leukotriene $B_4$ ($LTB_4$) levels in ears of CD-1 mouse as well as on spontaneous colorectal carcinogenesis in Min (Apc+/-) mice. Two doses of TFs, 0.18 and 0.71 mg, were topically applied to mice ears twice a day for 3.5 days (7 treatments) 20 mins prior to each TPA (0.4 nmol) treatment. These doses of TFs inhibited TPA-induced a) inflammation by 74 and 97%, b) up-expression of IL-1β protein levels by 93 and 99%, and c) up-expression of IL-6 protein levels by 58 and 99%, respectively. In these experiments, TFs (0.18 mg or 0.71 mg) also inhibited TPA







induced formation of $PGE_2$ by 27-100% and $LTB_4$ by 92-100%. In addition, oral administration of 0.2% or 0.4% TFs (28%) enriched black tea extract in drinking water as sole source of drinking fluid to female Min (Apc+/-) mice for 10 weeks inhibited the formation of the numbers of colorectal tumors per mouse by 39 or 29%, respectively. The percentage of mice bearing colorectal tumors was also inhibited by 39 or 29%, respectively. Furthermore, in these mice there was a reduction in the formation of the number of small intestinal tumors per mouse by 39 or 29% respectively.

Black tea accounts for about 80% of tea consumed worldwide (*1*). Black tea is produced from fresh tea leaves by fermentation through enzymatic oxidation (*1,2*). The hot water extract of black tea contains about a 2-6% mixture of theaflavin polyphenols, and greater than 20% thearubigens (*1-3*). The major theaflavin constituents of black tea are theaflavin (TF), theaflavin-3-gallate (TF-3-G), theaflavin-3'-gallate (TF-3'-G) and theaflavin-3,3'-digallate (TF-3,3'-diG). Black tea and green tea polyphenols have antioxidant activity (*4-6*) and the reported biological effects of black tea may be partially related to its antioxidant properties. Liang *et al.* (*5*) showed that topical application of the major constituents of black tea inhibited TPA-induced mouse ear edema. Additionally, black tea extract also be reported to have anti-inflammatory activity in the carrageenan-induced paw edema model in rats (*5*). Furthermore, the black tea constituent theaflavins were observed to inhibit TPA-induced edema of the mouse ear with the following order of potency where TF-3,3'-diG > TF-3-G = TF-3'-G > TF (*6*). Luceri *et al.* reported that administration of black tea extract equal to 40 mg of black tea polyphenols/kg/rat inhibited AOM-induced expression of COX-2, iNOS, glutathione *S*-transferase (GST), GST-M2 and GST-P in colon tumors (*7*). Pan *et al.* (*8*) reported that black tea polyphenols inhibited the activation of NFκB in activated murine macrophages (RAW 264.7 cell line). Theaflavin-3,3'-digallate (TF-3,3'-diG) is the most potent inhibitor of the activation of NFκB among black tea polyphenols (*9*). Recently, our laboratory demonstrated that topical application of TF and TF-3,3'-diG inhibited TPA-induced up-expression of IL-1β and IL-6 protein levels as well as inhibited TPA-induced increasing levels of $PGE_2$ and $LTB_4$ in mouse ear tissues (*6*). TF-2 (a mixture of TF-3-G and TF-3'-G) has been shown to inhibit COX-2 in cell lines (*10*). These mechanisms add strength to the claim that black tea may be an

anti-inflammatory agent by affecting the molecular targets that lead to inflammation. This report deals with the effect of black tea on TPA-induced inflammation, expression of pro-inflammatory cytokines, arachidonic acid metabolism in mouse ear and colon carcinogenesis in C57BL/6J Min (Apc+/-) mice providing further evidence to its mechanism of action.

## Materials and Methods

### Animals and Chemicals

Female CD-1 mice (3-4 weeks old) were purchased from the Charles River Breeding Laboratories (Kingston, NY). Female C57BL/6J Min (Apc+/-) mice were purchased from Jackson Laboratories (Bar Harbor, ME). Theaflavin related compounds (theaflavin mixture) were prepared as previously described (*11*). 12-*O*-Tetradecanoylphorbol-13-acetate (TPA) was purchased from Sigma Chemicals Company (St. Louis, MO). IL-1β and IL-6 ELISA kits were purchased from BioSource (Camarillo, CA). $PGE_2$ and $LTB_4$ EIA kits were purchased from Cayman (Ann Arbor, MI). Phosphate buffered saline was used to homogenize tissue samples.

*Animal treatment CD-1 mice*

Female CD-1 mice (23-30 days old, obtained from Charles River Breeding Laboratories, Kingston, NY) were divided into 3-4 groups of 5-6 mice each. All test compounds were dissolved in acetone, which served as the solvent (negative) control as it does not induce inflammation. A TPA control group was done to indicate the maximum induction of inflammation in mouse ear. Black tea theaflavin mixtures were evaluated in a dose dependent manner for their inhibitory effect on mouse ear inflammatory model.

*Topical application of theaflavins and TPA*

Both ears of CD-1 mice were treated topically with 15 μL acetone (solvent control group), TPA (TPA control) or test compound in acetone 20 mins before topical application of 15 μL acetone (solvent control) or 0.4 nmol TPA in acetone on the mouse ear. This treatment was continued twice a day for 3.5 days (7 treatments). Six hours after the last TPA treatment, the mice were sacrificed



by cervical dislocation and the ears punches (6-mm in diameter) were taken and weighed. The ear punches from each group were then combined and homogenized with phosphate buffered saline. The resulting homogenate was centrifuged and the supernatant was used to test for the levels of the various inflammatory mediators using the enzyme linked immunosorbent assay (ELISA).

*Oral administration of theaflavin*

Two doses, 5 and 10 mg of theaflavin were evaluated to examine the dose-response. For these experiments theaflavins were dissolved in water and given by gavages to CD-1 mice prior to TPA application. Each dose was given in two sequences. The 5 mg dose was given twice at 2.5 mg each time. Similarly, the 10 mg dose was given twice at 5 mg each. The total pretreatment time for theaflavin was 60 mins and these studies were design so that the first dose of theaflavin was given 60 mins and the second dose 20 mins prior to TPA application. At 6 hrs after TPA treatment, mice were sacrificed to evaluate ear inflammation.

*Female C57BL/6J Min (Apc+/-) mice*

For spontaneous colon carcinogenesis study, female C57BL/6J Min mice (APC+/-; 5-6 weeks old; 3 groups with 13 mice per group) were given water (control), 0.2% or 0.4% black tea in drinking water as sole source of drinking fluid for 10 weeks, for these experimental animals. The mice were then sacrificed for colorectal cancer evaluation.

### Synthesis of Theaflavin Mixtures

A mixture of theaflavins was synthesized from green tea polyphenols using enzymatic oxidation methods. Specifically, after filtration, the crude green tea polyphenol (1.8 g, commercial sample containing 80% catechins) was loaded directly onto a Sephadex LH-20 column eluted first with 95% ethanol to remove non-catechin flavonoids, and then the column was eluted with acetone to obtain a mixture of tea catechins (1.34 g). The tea catechins were dissolved in a pH-5 buffer (50 mL), which contained 4 mg horseradish peroxidase. While being stirred, 3.0 mL of 3.13% $H_2O_2$ was added 5 times during 1 hr. The enzymatic reaction solution containing catechins and crude peroxidase had turned into a reddish solution during oxidation reaction. The reaction mixture was extracted by ethyl acetate (50 mL × 3). After concentration, the residue (0.97 g) was

subjected to Sephadex LH 20 column eluted with acetone-water solvent system (from 35% to 50%). 350 mg of a theaflavin mixture was obtained (TF1: TF2: TF3 is 1:1:1; TF1 is theaflavin, TF2 is the mixture of theaflavin monogallates, TF3 is theaflavin 3,3'-digallate).

### Preparation of Ear Homogenates

Tissues were homogenized in a phosphate buffered saline solution containing 0.4 M NaCl, 0.05% Tween-20, 0.5% bovine serum albumin, 0.1 mM phenylmethylsulphonyl fluoride, 0.1 mM benzethonium, 10 mM EDTA and 20 mM KI aprotinin per mL. The homogenates were centrifuged at 12,000 x g for 60 mins at 4 °C. The supernatant was used for the determination of cytokine levels.

A two-site sandwich Enzyme-Linked ImmunoSorbant Assay (ELISA) was used to assay for cytokines.

### ELISA Assay Procedure

The IL-1β and IL-6 ELISA kits follow the same basic procedure. The capture antibody, diluted with PBS, was used to coat a 96-well plate overnight at room temperature. The plate was then washed, blocked (1% BSA, 5% sucrose in PBS with 0.05% $NaN_3$), and washed again. The standards were added to the plate leaving at least one zero concentration well and one blank well. The diluted samples (1:3-1:8) were then added to the plate. After incubating for 2 hrs the plates were washed and the detection antibody added. After incubating for another 2 hours the plates were washed and Streptavidin-HRP was added. After 20 mins incubating, the plates were washed and substrate ($H_2O_2$ and tetramethylbenzidine) was added. After another 20 mins incubating, the stop solution (2 N $H_2SO_4$) was added and the plates were read with a microplate reader at a wavelength of 450 nm.

The $LTB_4$ and $PGE_2$ ELISA kits follow the same basic procedure. The well plates are pre-coated with goat polyclonal anti-mouse IgG and blocking proteins. The standards and samples are added to the wells and incubated for an hour with tracer and anti-serum. After washing, Ellman's Reagent is added for color development. After incubating in the dark, the plate is read by microplate reader at a wavelength of 420 nm.



## Results

### Effect of Topical Application of Theaflavin Mixture (TFs) on TPA-induced Ear Inflammation Mouse Model:

Topical application of the two concentrations of TFs (either 0.18 or 0.71 mg) to both ears of CD-1 mice 20 mins before each TPA (0.4 nmol) treatment inhibited TPA induced ear inflammation by 74 and 97%, respectively (Panel A, Figure 1). In these experiments TPA-induced up-regulation and increased expression of IL-1β protein levels were inhibited by 93 or 99%, respectively (Panel B, Figure 1), and inhibited TPA-induced up-expression of IL-6 protein levels by 58 or 99%, respectively (Panel C, Figure 1). The results indicated that TFs strongly inhibited TPA-induced inflammation, up-expression of IL-1 and IL-6 protein levels in mouse ears.

### Effects of Topical Application of TFs on TPA-induced Arachidonic Acid Metabolism in Ears of CD-1 Mice

Effects of TFs on TPA-induced formation of arachidonic acid metabolites, $PGE_2$ and $LTB_4$ were evaluated in the mouse ears. PGE2 and LTB4 are formed by TPA-induced increase in metabolism of arachidonic acid (AA) via cyclooxygenase and lipoxygenase pathways, respectively. Results showed that there was a concentration dependent inhibition of formation of these metabolites when TF was topically applied to mice ears. $PGE_2$ levels were reduced by 27 and 100% (Panel A, Figure 2), while $LTB_4$ levels were down by 92 and 100%, respectively (Panel B, Figure 2). Although, TFs inhibited TPA-induced formation of AA metabolites, PGE2 and LTB4 from both cyclooxygenase and lipoxygenase pathways, our data suggests that TFs had a stronger inhibitory effect on the formation of AA metabolites from lipoxygenase pathway than the cyclooxygenase pathway.

### Inhibitory Effect of Oral Administration of TFs on TPA-induced Edema of Mouse Ears

Oral intubation of 2.5 or 5.0 mg of TFs to female CD-1 mice at 60 and 20 mins before topical application of TPA (1.5 nmol) inhibited TPA-induced edema of CD-1 mouse ears by 45 and 56%, respectively. Aspirin (2.5 mg), which is a known anti-inflammatory agent served as positive inhibitor control, inhibited TPA-induced edema of mouse ear by 35%. The inhibitory effect of both low dose (5 mg) and high dose (10 mg) of TFs and aspirin (2.5 mg) were statistically different from the positive control TPA (group 2).

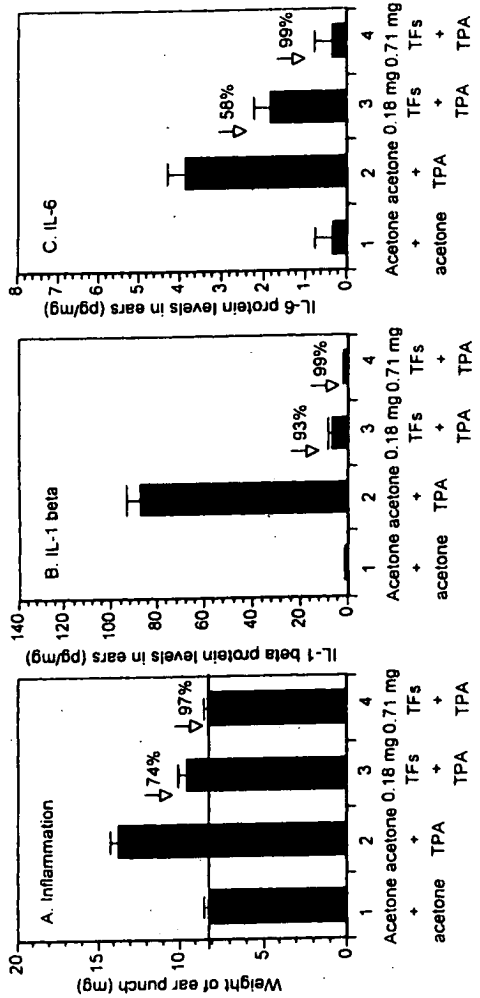



*Figure 1. Inhibitory effect of theaflavin mixture (TFs) on TPA-induced inflammation and pro-inflammatory cytokine IL-1β and IL-6 proteins in mouse ears. Both ears of female CD-1 mice were treated topically with acetone or TFs in acetone at 20 mins before application of TPA (0.4 nmol) twice a day for 3.5 days. The mice were killed at 5 hrs after the last dose of TPA treatment. Ear inflammation and pro-inflammatory cytokine proteins were determined.*



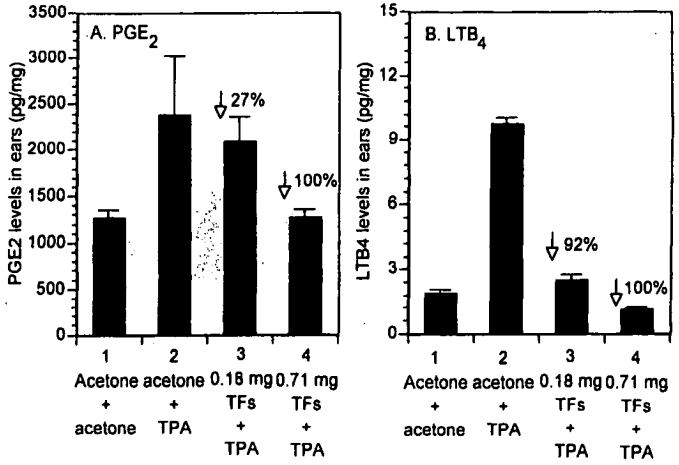
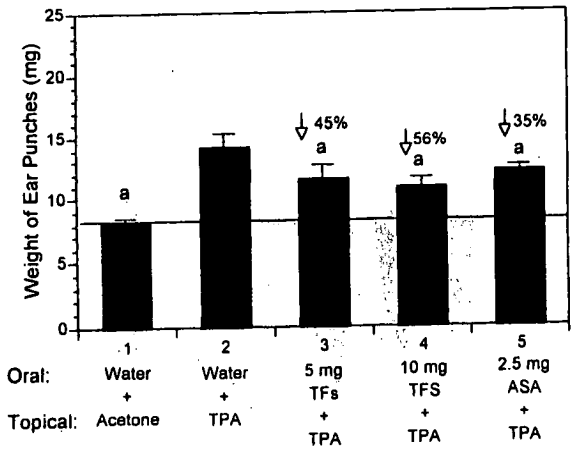



*Figure 2. Inhibitory effect of theaflavins (TFs) on TPA-induced formation of $PGE_2$ and $LBT_4$ levels in mouse ears. Both ears of female CD-1 mice were treated topically with acetone or TFs in acetone at 20 mins before application of TPA (0.4 nmol) twice a day for 3.5 days. The mice were killed at 5 hrs after the last dose of TPA treatment. $PGE_2$ and $LBT_4$ proteins were determined.*




[a] Statistically different from group 2 TPA ($P<0.05$) as determined by the Student's t test.

*Figure 3. Inhibitory effects of oral administration of theaflavin mixture (TFs) on TPA-induced ear inflammation in CD-1 mice, Female CD-1 mice were given 2.5 mg and 5.0 mg of TFs or aspirin (ASA, 1.25 mg) in 1 mL water by oral gavage at 60 and 20 mins before topical application of acetone or TPA (1.3 nmol) on both ears. The mice were killed at 6 hrs after TPA treatment. Ear punches (6-mm in diameter) were taken and weighed. Data are mean±SE from 12 ears average.*



## Inhibitory Effect of Oral Administration of Black Tea Extract (Enriched with Theaflavin Mixture) on Colorectal Carcinogenesis in Min (Apc+/-) Mice Model

Oral administration of 0.2% and 0.4% of black tea extract (28% TFs) as sole source of drinking fluid to female C57BL/6J Min (Apc+/-) mice inhibited the formation of the numbers of colorectal tumors per mouse by 59 or 34%, respectively. The percent of mice bearing colorectal tumors was inhibited by 56 or 29%, respectively (Figure 4). Oral administration of 0.2% and 0.4% of black tea extract enriched with theaflavin in water, the sole source of drinking fluid to female Min mice (Apc+/-) for 10 weeks inhibited formation of the numbers of small intestinal tumors per mouse by 39 and 29%, respectively. The total numbers of small and large intestinal tumors per mouse were inhibited by 39 and 29%, respectively (Figure 5).

There was no change in the body weight (water control group vs. black tea group.)

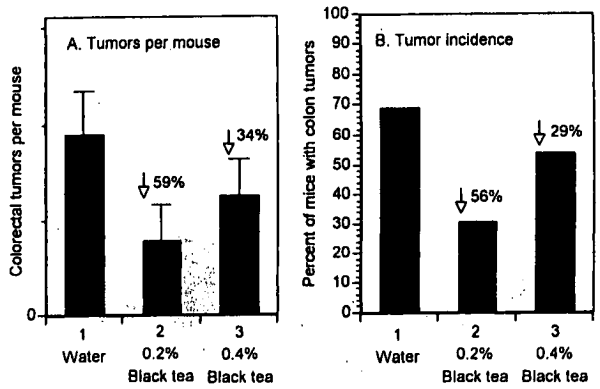



*Figure 4. Effect of oral administration of black tea extract (enriched with theaflavins) on formation of colorectal tumors in C57BL/6J Min mouse (Apc+/-). Female Min mouse (Apc+/-; 5-6 week old; 13 mice per group) were orally administered with water, 0.2% black tea and 0.4% black tea for 10 weeks. The mice were killed and corectal tumors were examined and counted. Data are expressed as the mean±SE as determined by Student's t test.*

## Discussion

Present results demonstrated that topical application of theaflavin mixture (TFs) strongly inhibited TPA-induced inflammation, up-expression of IL-1β and IL-6 protein levels in ears of CD-1 mice. Additionally, TFs also strongly inhibited TPA-induced formation of $PGE_2$ and $LTB_4$ levels in mouse ears, however, the inhibition of formation of $LTB_4$ was greater than that of $PGE_2$. These data suggested that TFs inhibited arachidonic acid metabolism by blocking the lipoxygenase pathway to a greater extent than the cyclooxygenase pathway. Thus, combination of TFs with sulindac (a cyclooxygenase inhibitor) showed some synergetic effect. Oral intubation of theaflavin mixture also inhibited TPA-induced ear edema (local inflammation). These observations suggested that theaflavin or its metabolites may be absorbed through the intestine and is transported to mouse ears. Feeding black tea extract enriched with theaflavin mixture (about 28%) in water, the sole source of drinking fluid, to female C57BL/6J Min (Apc+/-) for 10 weeks inhibited the formation of the numbers of small and large intestinal tumors per mouse. However, 0.4% black tea extract had less inhibitory effects on both numbers of small and large intestinal tumors per mouse. More studies are needed to determine the bioavailability and anti-carcinogenic effects of black tea and theaflavin rich black tea .

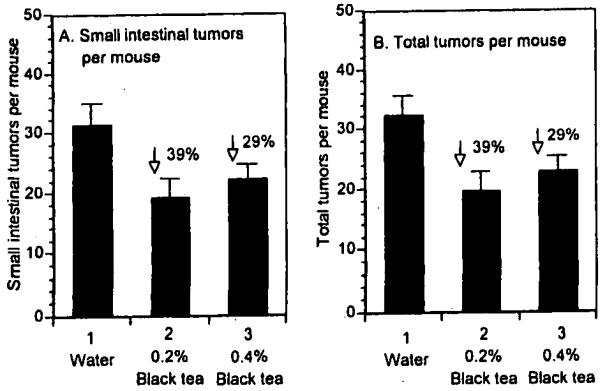



*Figure 4. Effect of oral administration of black tea extract (enriched with theaflavins) on formation of small and large intestinal tumors in C57BL/6J Min mouse (Apc+/-). Female Min mouse (Apc+/-; 5-6 week old; 13 mice per group) were orally administered with water, 0.2% black tea and 0.4% black tea for 10 weeks. The mice were killed and corectal tumors were examined and counted. Data are expressed as the mean±SE as determined by Student's t test.*



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# JAX® Mice Data Sheet

Go to JAX® Mice Query Form

## Strain Name: C57BL/6J-*Apc*$^{Min}$/J

## Stock Number: 002020



**General Terms and Conditions of Sale**

*Former Name* C57BL/6J-*Apc*$^{Min}$ (Changed: 03-dec-2003)

*Symbol* ***Apc*$^{Min}$;**

# Product Information

## Strain Details

| | |
|---|---|
| **Type** | JAX® GEMM® Strain - Chemically induced Mutation;<br>Additional information on JAX® GEMM® Strains. |
| **TJL Mating System** | Inbred x Heterozygote |
| **Investigator - Mutation Made By** | Dr. William F. Dove, University of Wisconsin |
| **Investigator - Donating** | Ms Fang Jin, Univ of California, San Francisco |
| **Backcross Generation** | N46 (03-jan-2001) |
| **Appearance** | black<br>Related genotype *a/a* |

**Strain Description**

The C57BL/6J-*Apc*$^{Min}$/J strain is a congenic derivative of C57BL/6J that is highly susceptible to spontaneous intestinal adenoma formation. Homozygous mice are not viable. It was initially reported that one hundred percent of the C57BL/6J-*Apc*$^{Min}$ heterozygous mice raised on a high fat diet develop in excess of 30 adenomas throughout the intestinal tract and most die by 120 days of age. Heterozygotes also develop anemia. (Moser *et al.*, 1990, Su *et al.*, 1992). A small number of C57BL/6J-*Apc*$^{Min}$ heterozygous female mice develop mammary tumors. A subsequent publication indicates that this strain may carry a dominant modifier (*Mom2*) gene that reduces the number and incidence of polyp formation in C57BL/6J-*Apc*$^{Min}$ heterozygous mice (Silverman *et al.*, 2002).

**Strain Development**
The *Min* mutation was discovered in the progeny of a C57BL/6J male mutagenized by ethylnitrosourea and an AKR/J female. One F1 female from the cross displayed circling behavior and was mated to a C57BL/6J male. Some progeny from this backcross developed adult onset anemia and intestinal adenomas. The circling behavior was determined to be a separate heritable trait and was eliminated through subsequent crosses to C57BL/6J. This strain was imported into The Jackson Laboratory in 1992.

## Gene Details

| | |
|---|---|
| **Symbol** | *Apc*$^{Min}$ |
| **Allele Name** | multiple intestinal neoplasia |
| **Gene Symbol and Name** | *Apc*, adenomatosis polyposis coli |
| **Chromosome** | 18 |
| ***Gene Common Name (s)*** | Min; |
| **Symbol Description** | The *Apc*$^{Min}$ allele has a T to A transversion at nucleotide 2549. This point mutation changes codon 850 to a pre-mature stop codon. (Su et al, 1992.) This is an autosomal dominant mutation.<br>The *Apc* gene is the homolog of human APC (adenomatous polyposis coli) gene.<br>Mouse Locus Catalog entry |

## Control Information

| Symbol | Control |
|---|---|
| *Apc*$^{Min}$ | Wildtype from the colony |
| *Apc*$^{Min}$ | C57BL/6J 000664 |

**Control Notes** Wildtype mice from the colony or C57BL/6J mice (Stock No. 000664) may be used as controls.
Considerations for Choosing Controls
Control Pricing Information for JAX® GEMM® Strains

## Genotyping Protocols

ApcMin

## Colony Maintenance

| | |
|---|---|
| **Breeding and Husbandry** | This strain is maintained by breeding heterozygotes males to C57BL/6J females. Female heterozygotes are not recommended because anemia and intestinal adenomas interfere with pregnancy. Breeding performance in heterozygote males declines as anemia and tumors develop. |
| **Diet Information** | LabDiet® 5K20 |

## Related Strains

## Additional Web Information

Genetic Quality Control Annual Report
Genetic Quality Control Annual Report
JAX Notes, Fall 1993; 455. The C57BL/6J-*Min*/+ Mouse.

## Animal Health Reports

**Room Number** AX1

## Research Applications

This mouse can be used to support research in many areas including:

*Apc$^{Min}$ related*

**Cancer Research**
Increased Tumor Incidence (Mammary Gland Tumors)
Increased Tumor Incidence (Adenomas: intestinal adenomas)

**Mouse/Human Gene Homologs**
adenomatosis polyposis coli

## References

***Primary Reference***

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Additional References

# Price and Supply Information

Strain Name: **C57BL/6J-*Apc$^{Min}$*/J**
Stock Number: **002020**

## Price Details

**Prices are based on shipping destination. To view prices, select your shipping destination.**

- USA, Canada or Mexico
- International Destinations (EXCEPT Canada and Mexico)

## Supply Details

| | |
|---|---|
| **Standard Supply** | **Level 3.** Colony sized for average order of 5-15 mice. Larger quantities or custom orders arranged upon request. Age range recommended. |
| **Supply Notes** | Shipped at a specific age in weeks. Mice at a precise age in days, littermates and retired breeders are also available.<br>Strains that must be genotyped are not available until five to seven weeks of age.<br>This strain is included in the Induced Mutant Resource collection. |
| **Licensing** | See **General Terms and Conditions of Sale** below. |
| **Control Information** | View Control Information in Strain Details.<br>View Control Pricing Information for JAX® GEMM® Strains. |

# General Terms and Conditions of Sale

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## The Jackson Laboratory's Genotype Promise

The Jackson Laboratory has rigorous genetic quality control and mutant gene genotyping programs to ensure the genetic background of JAX® Mice strains as well as the genotypes of strains with identified molecular mutations. JAX® Mice strains are only made available to researchers after meeting our standards. However, the phenotype of each strain may not be fully characterized and/or captured in the strain data sheets. **Therefore, we cannot guarantee a strain's phenotype will meet all expectations.** To ensure that JAX® Mice will meet the needs of individual research projects or when requesting a strain that is new to your research, we suggest ordering and performing tests on a small number of mice to determine suitability for your particular project.

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# JAX® Mice Data Sheet

## Strain Name: C57BL/6J-*Apc*$^{Min}$/J

## Stock Number: 002020

**Link to main data sheet for 002020.**

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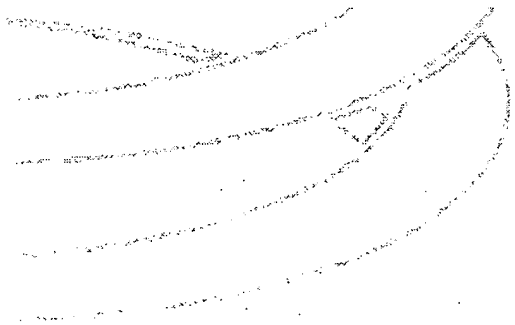

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# Alteration of Gene Expression in Normal-Appearing Colon Mucosa of $APC^{min}$ Mice and Human Cancer Patients

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## ABSTRACT

**The expression of many genes is altered in colon cancer, but the roles of these genes in carcinogenesis are unclear. Using real-time quantitative PCR, we demonstrated that several genes previously implicated in human colon cancer undergo altered expression in the $APC^{min}$ mouse adenomatous polyp, a precursor of cancer, as well as in normal-appearing surrounding mucosa. The five genes that were most highly up-regulated in mouse polyp were also significantly up-regulated in polyp-free colon mucosa. Similar changes occurred in morphologically normal mucosa of surgical sections taken from human cancer patients, frequently extending to the margins. Thus, morphologically normal colon mucosa in $APC^{min}$ mice and in human cancer patients is not metabolically normal. Altered gene expression in this tissue does not appear to result from a field effect because there was no correlation between extent of altered regulation and distance from polyp or tumor. Our data suggest that alterations of expression levels of these genes may be an early event in carcinogenesis and a marker of risk for the development of colon cancer.**

## INTRODUCTION

Colon cancer is thought to result from a series of mutations and other genetic derangements that result in pathological changes in key metabolic pathways within the cell. Large-scale screening of gene expression profiles of colon cancers, using such methods as cDNA arrays or reverse transcription-PCR, have identified many of these alterations (1–6). However, screening of advanced carcinomas cannot distinguish changes in gene expression that are critical to the process of carcinogenesis from those that result from the progressive derangement of the genome and accompanying disruption of large metabolic networks.

One approach to identifying the essential metabolic events targeted in cancer is to manipulate certain genes in colon cancer cell lines and determine the effects on proliferation, invasiveness, and other properties of these cells (7, 8). However, the success of this approach depends on selecting, from the potentially large number of candidates identified by large-scale screening, a relatively small number of promising genes. Moreover, altered expression of a particular gene may have effects in a tumor cell line without being involved in carcinogenesis *in vivo*. An alternative approach is to identify metabolic events that are altered early in carcinogenesis by screening precancerous tissue (1, 9, 10). Most colon cancers begin as an adenomatous polyp, which may progress to a carcinoma as a result of the accumulation of genetic alterations. Therefore, screening at this stage may provide greater insight into cancer pathogenesis.

The $APC^{min}$ mouse carries a mutation in the adenomatous polyposis coli (*APC*) gene. As in humans, this mutation leads to the early development of intestinal adenomas that can progress to locally invasive carcinomas (11). This makes it possible to screen morphologically normal tissue from these animals for the expression of genes that may be involved in carcinogenesis, with colon tissue from wild-type animals providing a control.

We hypothesized that some of the metabolic alterations observed in colon carcinomas occur early in carcinogenesis, *i.e.*, before morphological alterations are apparent. To test this hypothesis, we screened a panel of genes to determine whether some have altered expression levels at earlier stages of carcinogenesis. We screened a panel of 15 genes that are altered (up- or down-regulated) in the late stages of human colon cancers. These genes function in several pathways related to cancer development, including the APC/β-catenin pathway, the nuclear factor-κB pathway, cell cycle, and inflammation, and therefore may represent the much larger set of genes that are altered in colon cancer.

We report here that we identified several candidate genes that have altered expression levels in adenomatous polyps as well as in morphologically normal colon mucosa from $APC^{min}$ mice. We also found altered expression levels of some of these same genes in apparently normal mucosa from human colon cancer patients. Our findings suggest that altered expression of these genes may be a useful indicator of the presence of colon cancer and may aid in identifying patients at risk of developing cancer.

## MATERIALS AND METHODS

**Animals.** C57BL/6J-min/+ mice and their wild-type littermates were obtained from The Jackson Laboratory (Bar Harbor, ME). The *APC* genotype was confirmed by use of primers specific for the mutated *APC* gene.

**Human Subjects.** Samples of colon cancer and adjacent grossly normal-appearing tissue were obtained at the time of surgery from patients undergoing colon surgical resection at California Pacific Medical Center (San Francisco, CA). We also obtained biopsies of grossly normal-appearing colon tissue from patients with no adenomatous polyps and no known family history or previous colon cancer who had submitted to routine colonoscopic examination. Patients ranged in age from 50–83 years and included both males and females. The research protocols for removal of both surgical samples and normal biopsies were approved by the California Pacific Medical Center Institutional Review Board. The appropriate procedure for obtaining informed consent was followed for all individuals participating in these studies.

**Preparation of Mouse Colonic Mucosal Cells.** The entire colon was removed from the $APC^{min}$ and wild-type mice, opened longitudinally, and washed extensively in cold PBS. Any visible adenomatous polyps were removed completely and analyzed. The colon was then divided into six equal segments from the proximal to the distal end. Colonic mucosa samples were isolated from each segment by 2 min of vigorous vortexing of the segment in 1 ml of cold PBS and centrifugation at 40 × *g* for 5 min.

**Preparation of Human Colonic Mucosal Cells.** Colon cancer and adjacent grossly normal-appearing mucosa (4–20 samples/patient) were obtained from patients at the time of segmental resection to remove colon cancer. Control samples consisted of biopsies (six to eight from the rectosigmoid and three to four from the ascending colon per patient) obtained in the course of colonoscopic examination of patients with no personal or family history of colon cancer and with no polyps. For the surgical samples, the easily separable mucosal layer from multiple sites of the adjacent normal tissue was lifted and dissected from the submucosal layer. All samples were snap-frozen on dry ice as soon as possible within 30 min of surgery, or 1 min of biopsy, and then were taken immediately to the laboratory for RNA preparation (see below).

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**Extraction and Preparation of RNA.** Total RNA was extracted from the individual adenomatous polyps and the isolated normal mucosal cells with use of RNAeasy kits from Qiagen (Valencia, CA). RNA samples were treated with RNase-free DNase to remove any genomic DNA contamination and were reverse-transcribed using Superscript II (Invitrogen, Carlsbad, CA) with oligo(dT) and random primers (Invitrogen). Fifty ng of cDNA from each sample were used as template for PCR amplification with specific oligonucleotide primers in the Applied Biosystems 5700 Sequence Detection System (PE Applied Biosystems, Foster City, CA). PCR reactions were performed according to the manufacturer's instructions, using the SYBR Green PCR Core Kit (PE Applied Biosystems). The identities of the PCR products were confirmed by melting temperatures and dissociation curves.

**Analysis of Gene Expression.** We analyzed 15 genes, all of which have previously been shown to be altered in expression in human colon cancer. They fall into four groups, including those involved in the (*a*) APC/β-catenin pathway, including c-myc, cyclin D1, and proliferating peroxisome activating receptor-α (PPARα; Refs. 12, 13); (*b*) nuclear factor-κB/inflammation pathway, including growth-related oncogene-α (Gro-α), osteopontin (OPN), macrophage-colony-stimulating factor (MCSF-1; Ref. 9), cyclooxygenase-1 (COX-1) and -2 (COX-2), Gro-γ [or its mouse homolog, macrophage inflammatory protein-2 (MIP-2)], interleukin-8 [IL-8; or its mouse homolog, stroma-derived factor (SDF-1)], and CXC cytokine receptor 2 (CXCR2); (*c*) cell cycle/transcription factors, including $p21^{cip/waf1}$, cyclin D1, c-myc, and PPARα, -δ, and -γ (1, 14); and (*d*) cell communication signals, including IL-8, PPARα and -γ, CXCR2, CD44, and OPN. Most of these genes have been reported to be up-regulated in human colon cancers, although some, such as $p21^{cip/waf1}$, are down-regulated.

Specific primers against each gene were designed with the Primer Express Software (PE Applied Biosystems). Primer length was 21–27 nucleotides, with a theoretical melting temperature of 58–60°C. Sizes of amplicons ranged from 66 to 150 bp. Primers were designed to amplify only cDNA template but not genomic DNA template whenever possible. The specificities of the primers used were demonstrated by the appearance of a single product on 10% PAGE and a single dissociation curve of the PCR product.

All of the cDNA samples were tested for genomic DNA contamination by use of primers for β-actin genomic DNA. With these primers, PCR products derived from the genomic DNA have a different melting temperature and length from the PCR product derived from cDNA. Only cDNA samples without genomic DNA contamination were used.

For quantitation of gene expression, the fluorescence of the SYBR Green dye bound to the PCR products was measured after each cycle, and the cycle numbers were recorded when the accumulated signals crossed an arbitrary threshold ($C_T$ value). To normalize this value, a $\Delta C_T$ value was determined as the difference between the $C_T$ value for each gene and the $C_T$ value for β-actin or histidyl tRNA synthetase (his-tRNA synthetase; Ref. 3). All PCR reactions were performed in duplicate when cDNA samples were available. The average $\Delta C_T$ values were used in the following analysis. In addition, PCR for β-actin or his-tRNA synthetase were repeated in each experiment as references and were shown not to vary significantly under the different experimental conditions used in this study. For each gene, a $\Delta\Delta C_T$ value was determined as the difference between the $\Delta C_T$ value for each individual sample and the average $\Delta C_T$ value for this gene obtained from the control samples. These $\Delta\Delta C_T$ values were then used to calculate relative gene expression values as described. (Applied Biosystems; User Bulletin 2; December 11, 1997). Because we had limited amounts of RNA from human biopsies, his-tRNA synthetase was assayed only in approximately half of the samples. The results indicated that expression of both β-actin and his-tRNA synthetase did not vary between the cancer patient group and the control group. Therefore, calculations of $\Delta\Delta C_T$ and relative gene expression values yielded similar results when we used either β-actin or his-tRNA synthetase as reference. The results of statistical analyses were obtained with β-actin as the reference.

**COX-2 Activity Assay.** Colonic mucosal cells were prepared as described above. The cells were homogenized in 0.1 M Tris-HCl (pH 7.8) in the presence of 1 mM EDTA. COX activity was assayed with the COX activity assay kit from Cayman Chemical (Ann Arbor, MI), which measures the peroxidase activity component of the COXs by colorimetric monitoring of oxidized *N,N,N',N'*-tetramethyl-*p*-phenylenediamine at 590 nm. The COX-1-specific inhibitor was included in the assay to obtain COX-2 specific activity. Assays were carried out in duplicate with 200 μg of protein.

**Immunohistochemical Analysis.** Five-μm sections of formalin-fixed, paraffin-embedded mouse colonic tissues were analyzed by immunostaining according to previously published methods (15). After deparaffinization and antigen retrieval with 10 mM citrate buffer (pH 6.0), sections were blocked in 1% normal sheep serum for 1 h and then incubated for 60 min at room temperature with the following antisera: 1:600 dilution of rabbit antimouse COX-2 antiserum from Cayman Chemical; 1:300 dilution of rabbit antimouse CXCR2 (IL-8RB); 1:300 dilution of goat antimouse OPN antiserum; or 1:300 dilution of goat antimouse Gro-α antiserum from Santa Cruz Biotechnology (Santa Cruz, CA). After washing, slides were incubated with biotinylated goat antirabbit or rabbit antigoat secondary antibody at a 1:300 dilution (DAKO, Carpenteria, CA), followed by streptavidin-horseradish peroxidase at a 1:300 dilution (Amersham, Arlington, IL). Color was developed with a 3,3'-diaminobenzidine tetrahydrochloride peroxidase substrate kit (Vector Labs, Burlingame, CA). Sections were counterstained with Mayer's hematoxylin and mounted with Permount (Fisher Scientific, Santa Clara, CA).

**Statistical Analysis.** In this study, expression patterns of several genes were compared between $APC^{min}$ mice/cancer patients *versus* controls. Rather than testing the expression of each gene separately and adjusting for multiple comparisons by methods that reduce statistical power, we tested expression patterns of all genes by multivariate ANOVA, a global test that accounts for correlations among expression levels. If the global test was significant, indicating that there was evidence that the overall expression patterns differed, we used univariate *t* tests to determine which genes were contributing to the global difference. All multivariate ANOVA tests were based on Wilks' λ criterion and were carried out on log(base 2) values for the expression because this transformation was required to achieve normal distribution of values.

## RESULTS

**Altered Gene Expression in Adenomatous Polyps from $APC^{min}$ Mice.** $APC^{min}$ mice (ages 6–23 weeks) had polyps ranging from 1 to 4 mm in both the colon and small intestine. Typically, ≥30 polyps were present in the small intestine and 0–3 in the colon of each mouse. In general, more polyps were found in older mice than younger ones. A total of 14 colonic polyps from eight mice were analyzed. All were adenomatous polyps. These were classified as low-grade dysplastic based on glandular architecture; nuclear hyperchromasia, stratification, and pleomorphism; and cytoplasmic mucus content. Table 1 lists the relative expression levels of all genes that were examined in these polyps relative to expression in colon mucosa from wild-type mice. A wide range of expression levels was observed;

Table 1 *Relative gene expression levels in colon polyps of $APC^{min}$ mice (mean ± SE)*

Gene expression levels were determined as described in the "Materials and Methods." In nos. 1~5, $n = 13$ in the wild-type littermate group, and $n = 14$ in the individual polyp group; in nos. 6~15, $n = 6$ in the wild-type littermate group, and $n = 10$ in the individual polyp group. Significance was determined by *t* test.

| No. | Gene | Wild-type littermate | Individual polyp | *P* |
|---|---|---|---|---|
| 1 | OPN[a] | 1.62 ± 0.60 | 430 ± 125 | <0.01 |
| 2 | MIP-2 | 1.74 ± 1.60 | 203 ± 43 | <0.001 |
| 3 | Gro-α | 1.40 ± 0.32 | 122 ± 19 | <0.001 |
| 4 | CXCR2 | 1.41 ± 0.35 | 105 ± 23 | <0.001 |
| 5 | COX-2 | 1.41 ± 0.25 | 82 ± 16 | <0.001 |
| 6 | Cyclin D1 | 1.34 ± 0.34 | 19 ± 3 | <0.001 |
| 7 | SDF-1 | 1.23 ± 0.34 | 11 ± 2 | <0.01 |
| 8 | c-myc | 1.09 ± 0.18 | 6.49 ± 0.96 | <0.001 |
| 9 | MCSF-1 | 1.05 ± 0.15 | 4.26 ± 1.60 | NS |
| 10 | CD44V6 | 1.17 ± 0.28 | 3.78 ± 0.61 | <0.01 |
| 11 | COX-1 | 1.07 ± 0.15 | 3.24 ± 0.60 | <0.01 |
| 12 | PPAR-γ | 1.13 ± 0.22 | 0.86 ± 0.24 | NS |
| 13 | $p21^{cip/waf1}$ | 1.11 ± 0.17 | 0.51 ± 0.07 | <0.05 |
| 14 | PPAR-δ | 1.16 ± 0.27 | 0.44 ± 0.05 | <0.05 |
| 15 | PPAR-α | 1.04 ± 0.12 | 0.17 ± 0.03 | <0.001 |

[a] OPN, osteopontin; MIP-2, macrophage inflammatory protein-2; Gro-α, growth-related oncogene-α; CXCR2, CXC cytokine receptor 2; COX-1 and -2, cyclooxygenase-1 and -2; SDF-1, stroma-derived factor-1; MCSF-1, macrophage-colony-stimulating factor; NS, not significant; PPAR-α, -δ, and -γ, proliferating peroxisome-activating receptor-α, -δ, and -γ.

several genes were up-regulated markedly, whereas others were down-regulated. Five genes—COX-2, GRO-α, CXCR2, OPN, and MIP-2, had a particularly high degree of altered expression in the polyps. All of these genes are up-regulated in human colon cancer or other cancers, although not to such a high degree (1, 8, 16, 17).

**Altered Gene Expression in Morphologically Normal Colon of $APC^{min}$ Mice.** We next analyzed the expression of these five genes in morphologically normal colon tissue from $APC^{min}$ mice at three different ages—6, 13, and 23 weeks—compared with normal colon tissue from wild-type littermates. After we removed all of the polyps from the $APC^{min}$ mice, we divided the polyp-free colons of the $APC^{min}$ mice and the age-matched wild-type littermates into six equal segments ~1.5 cm in length. Colonic mucosa was isolated as described in "Materials and Methods," and the expression of the five genes was analyzed.

In Fig. 1, each point represents the level of expression of a single gene in the mucosa of a single colon segment of a single mouse. Although the expression levels by segment and age showed little variability among wild-type animals, there was considerable variation among the $APC^{min}$ mice. To determine the significance of the differences between these latter values and those for wild-type animals, we applied multivariate analysis on the data presented in Fig. 1. In each age group, some genes were significantly up-regulated in at least some segments in $APC^{min}$ mice ($P < 0.01$). As shown in Table 2, all of these genes except OPN were significantly up-regulated in at least some segments of 23-week-old $APC^{min}$ mice, particularly in segment 6.

The distribution of these metabolic alterations was not correlated with the presence of polyps. As shown in Fig. 1*C*, in the 23-week-old $APC^{min}$ mice, a total of four polyps were detected in segments 1 and 2, one in segment 5, and none in the other segments; however, the greatest degree of up-regulation for most of these genes was observed in segment 6. This finding suggests that the observed changes in gene expression were not the result of a field effect caused by escape of altered cells from the polyps, but were intrinsic to the morphologically normal cells of the colon where they were detected.

**Expression of COX-2 Protein Was Elevated in Grossly Normal Colon Mucosa of $APC^{min}$ Mice.** To determine whether the up-regulation of RNA expression in the morphologically normal colon mucosa of $APC^{min}$ mice also resulted in protein overexpression, we assayed COX-2 activity in the grossly normal mucosa from six colonic segments in both $APC^{min}$ mice and the wild-type littermates. As illustrated in Fig. 2, COX-2 activity was elevated 5–8-fold in the grossly normal mucosa of 23-week-old $APC^{min}$ mice ($P < 0.05$ for segments 1, 2, 5, and 6, Mann–Whitney rank-order test).

To determine the cellular locations of the up-regulation of these genes, we performed immunohistochemical analysis of Gro-α, OPN, CXCR2, and COX-2 in the grossly normal colon mucosa from segments 1, 3, and 5 of two 23-week-old $APC^{min}$ mice and two age-matched wild-type mice. A total of nine aberrant crypt foci (ACF) were found in the samples from $APC^{min}$ mice (three in segment 1, one in segment 3, and five in segments 5 of the two $APC^{min}$ mice). No ACF were found in the samples from wild-type mice. Fig. 3 shows representative immunostaining results for COX-2. We did not detect COX-2 staining in the mucosa of wild-type mice (Fig. 3*A*). However, in adenomatous polyps from $APC^{min}$ mice, we observed strong staining in the macrophages and moderate staining in the epithelial cells (Fig. 3*D*). Macroscopically normal mucosa of $APC^{min}$ mice are shown in Fig. 3, *B* and *C*. Notably, COX-2 expression was prominent in macrophages from $APC^{min}$ mice (Fig. 3*B*). Weak COX-2 staining was observed in the epithelial cells of all of the ACF from $APC^{min}$ mice (Fig. 3*C*). However, in all samples analyzed, the epithelial cells of the normal crypt foci from $APC^{min}$ mice exhibited very weak or unde-

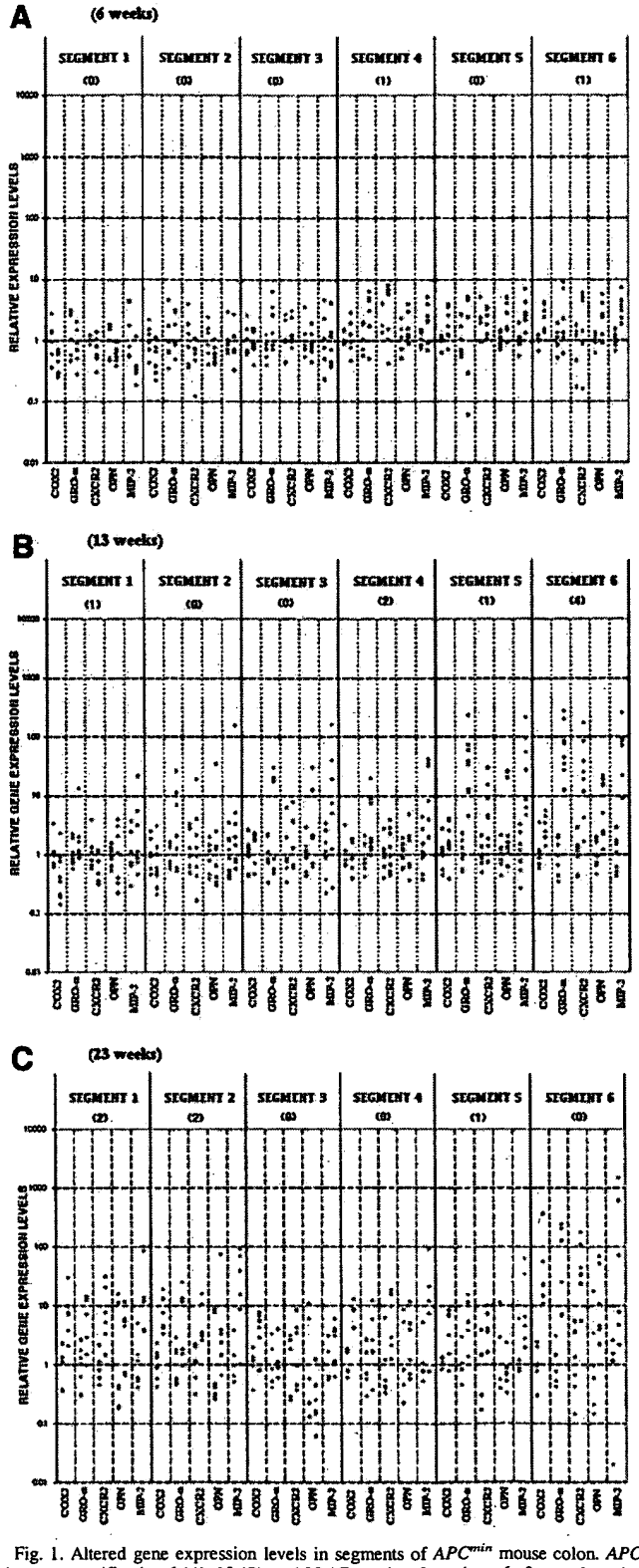


Fig. 1. Altered gene expression levels in segments of $APC^{min}$ mouse colon. $APC^{min}$ mice were sacrificed at 6 (*A*), 13 (*B*), and 23 (*C*) weeks of age ($n = 6–8$ at each age), and the colons were removed and divided into six segments from the most proximal (*segment 1*) portion to the rectum (*segment 6*). Mucosal tissue was removed from each segment, and total RNA was extracted from each sample. Expression levels of the indicated genes were determined by reverse transcription-PCR with specific primers for each gene, as described in the "Materials and Methods," and compared with a β-actin control. Relative expression levels were obtained by comparing the changes in threshold cycle values for each gene with the mean value of that gene for all wild-type animals ($n = 6–8$). Each *dot* represents the relative expression level of one gene from one wild-type (*red dots*) or $APC^{min}$ (*blue dot*) mouse. The total number of polyps found in all of the mice of a particular age for each segment is shown in *parentheses* at the *top* of the figure. *COX2*, cyclooxygenase-2; *GRO-α*, growth-related oncogene-α; *CXCR2*, CXC cytokine receptor 2; *OPN*, osteopontin; *MIP-2*, macrophage inflammatory protein-2.

Table 2 *Multivariate analysis of gene expression in normal-appearing colon mucosa of 23-week-old $APC^{min}$ mice compared with colon mucosa of wild-type mice*

Colons were removed from animals, and any polyps were removed. The colons were then divided into six segments, the colon mucosa was isolated, and gene expression was determined as described in the "Materials and Methods," with values for $APC^{min}$ mice compared with those for wild-type mice. Multivariate analysis was performed on these values as described in the "Materials and Methods," in which the significance of the difference in expression between $APC^{min}$ and wild-type mice was determined for each gene in the presence of all of the other genes. For this analysis, six mice were used for each group ($APC^{min}$ and wild-type), and one mucosa sample was analyzed per segment per mouse.

| | Colon segment[a] | | | | | |
|---|---|---|---|---|---|---|
| Gene | 1 | 2 | 3 | 4 | 5 | 6 |
| Cox-2[b] | ++ | ++++ | + | ++++ | + | +++ |
| CXCR2 | + | ++ | − | ++ | + | ++++ |
| MIP-2 | ++ | ++ | − | ++ | + | +++ |
| Gro-α | − | + | − | − | + | +++ |
| OPN | − | − | − | − | − | − |

[a] −, $P > 0.05$; +, $P < 0.05$; ++, $P < 0.01$; +++, $P < 0.001$; ++++, $P < 0.0001$.
[b] Cox-2, cyclooxygenase-2; CXCR2, CXC cytokine receptor-2; MIP-2, macrophage inflammatory protein-2; Gro-α, growth-related oncogene-α; OPN, osteopontin.

tectable Cox-2 staining (Fig. 3*B*). We were unable to demonstrate unequivocal staining for Gro-α, OPN, and CXCR2 (data not shown).

**Altered Gene Expression in Normal Colon Mucosa Adjacent to Carcinoma in Human Patients.** We next analyzed colon samples from human patients who had undergone surgery to remove colon carcinomas. Although all of the genes we analyzed in mice are differentially regulated (up- or down-regulated) in human colon cancers, previous studies have generally assumed that morphologically normal colon mucosa adjacent to the tumor is metabolically normal and have used such tissue as a baseline for comparison. Because of our findings with $APC^{min}$ mice, we were interested in determining whether this assumption is valid or whether there are altered gene expression profiles in morphologically normal colon mucosa from cancer patients. We therefore compared gene expression levels in morphologically normal-appearing colon mucosa from cancer patients with those in mucosa from patients without cancer. We analyzed two sets of data, one set consisting of samples from patients with cancer in the sigmoidal-rectal region, the other from patients with cancer in the ascending colon. In both studies, we examined expression levels of the same 15 genes that were analyzed in $APC^{min}$ mice, except for Gro-γ, the human analog of MIP-2 in mice, and IL-8, a close relative of SDF-1 in mice.

In both studies, the values obtained from the cancer patients were highly variable, much more so than the corresponding values from the controls (Table 3). This finding parallels the observations we made in $APC^{min}$ and wild-type mice, although the variation in humans was even higher. Despite the great variability, expression levels for several genes were much higher in some samples from cancer patients than for any samples from controls. For example, four of the genes that were significantly up-regulated in normal-appearing mucosa of $APC^{min}$ mice—CXCR2, GRO-α, COX-2, and OPN—were up-regulated in normal-appearing mucosa from some cancer patients to levels 50–200 times greater than those in controls. In addition, in some cancer patients, PPARα, -δ, and -γ were down-regulated 50–100-fold.

To evaluate the significance of these differences, we conducted a series of multivariate tests. In this analysis, seven genes were significantly up-regulated in morphologically normal mucosa from patients with sigmoidal-rectal cancer relative to controls: MCSF-1, OPN, IL-8, COX-2, CXCR2, p21, and CD44. Two genes—PPARδ and -γ—were significantly down-regulated (Table 3). Similar results were obtained for the ascending colon. Six of the seven genes significantly up-regulated in sigmoidal-rectal mucosa were also up-regulated in the ascending colon—MCSF-1, OPN, IL-8, COX-2, CXCR2, and CD44—along with COX-1. Likewise, PPARδ and -γ were significantly down-regulated in ascending colon (Table 3).

The difference between cancer patients and controls was even more striking when the relative expression levels of three of the most up-regulated genes—COX-2, OPN, and MCSF-1—were considered together. In Fig. 4, the log(base 2) of expression level of each of these three genes in each patient sample is plotted in a three-dimensional set of axes. In virtually every sample from a cancer patient, at least one of these three genes was significantly up-regulated relative to its expression level in any sample from a control. This analysis thus suggests that expression levels of these three genes considered together may be sufficient to distinguish normal colon mucosa in colon cancer patients from colon mucosa in controls.

**Altered Gene Expression in Normal Mucosa Adjacent to Carcinomas Is Not a Result of Field Effect.** The samples of normal-appearing mucosa from cancer patients were taken randomly from all areas of the surgical section. The distribution of samples taken from a single cancer patient is shown in Fig. 5, which indicates the approximate expression level in each sample of COX-2 and OPN. There was no correlation of expression level with distance from the cancer. Similar results were obtained with other, differently regulated genes (data not shown). These observations suggest that the differently regulated areas of gene expression in normal-appearing colon mucosa of cancer patients did not result from a field effect of cells spreading from the original cancer.

## DISCUSSION

The major goal of this study was to identify metabolic alterations that precede overt colon cancer and that might occur early in carci-

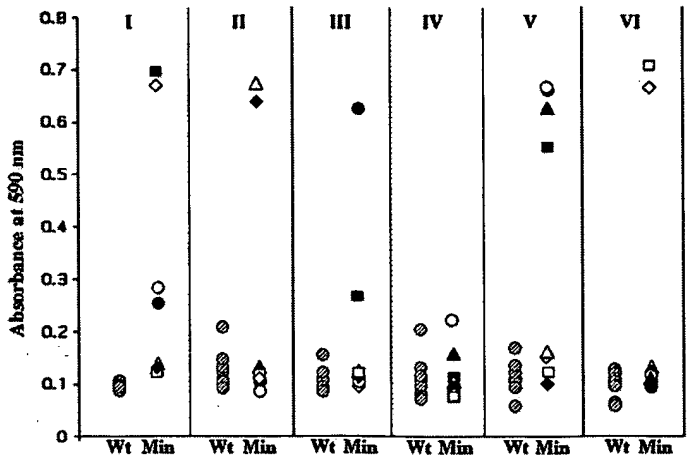


Fig. 2. Cyclooxygenase-2 enzymatic activity in the normal-appearing colonic mucosa of $APC^{min}$ mice. $APC^{min}$ mice (*Min*) and the wild-type littermates (*Wt*) were sacrificed at 23 weeks of age ($n = 8$ for each group). The *hatched circles* represent wild-type mice; the *other symbols* represent individual $APC^{min}$ mice. In $APC^{min}$ mice, the same symbol in different segments represents mucosa cells from the same $APC^{min}$ mouse. Colonic mucosa cells were obtained as described in "Materials and Methods." Cells were homogenized, and 200 μg of protein were used for each cyclooxygenase-2 enzymatic activity assay. The *Roman numerals* indicate the segment numbers.

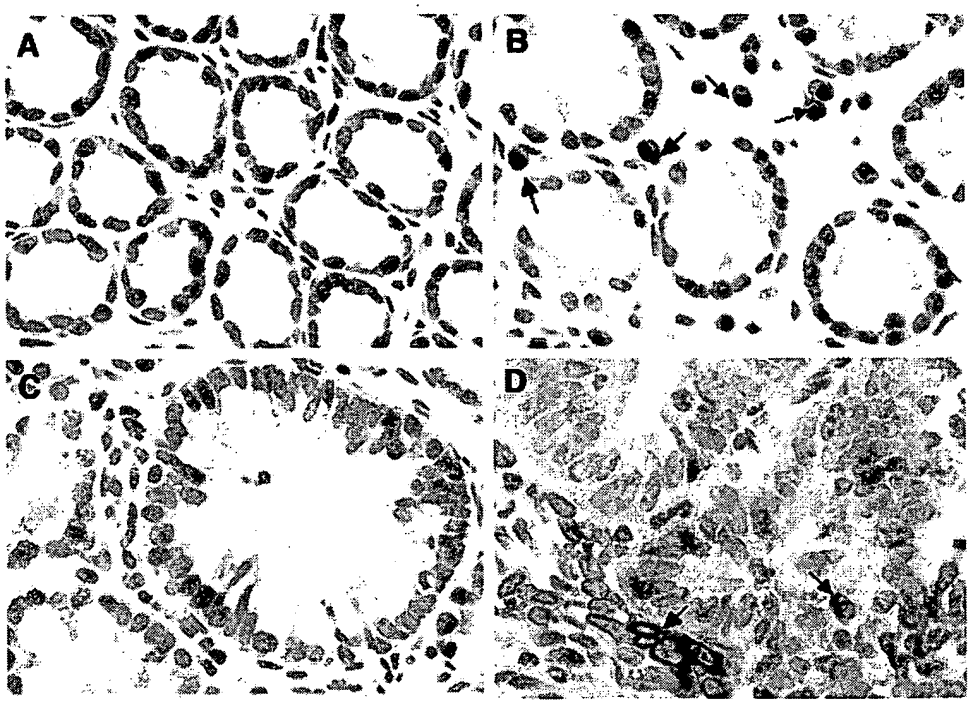


Fig. 3. Immunohistochemical analysis of cyclooxygenase-2 protein expression in grossly normal colonic mucosa of $APC^{min}$ mice. Representative sections of formalin-fixed, paraffin-embedded tissues from macroscopically normal colonic mucosa from a wild-type mouse (*A*) and a $APC^{min}$ mouse (*B* and *C*), and colonic adenomatous polyp tissue from a $APC^{min}$ mouse (*D*) were stained with anti-cyclooxygenase-2 antibody by the immunoperoxidase method. Nuclei were counterstained with Mayer's hematoxylin. *Arrows* in *B* and *D* indicate strongly stained macrophages. *Panel C* illustrates the presence of aberrant crypt foci in the macroscopically normal colonic mucosa of an $APC^{min}$ mouse. All photographs were taken at a ×40 magnification.

nogenesis. Our aim was not to identify all possible alterations but to analyze a representative sample. Accordingly, we selected 15 genes from four groups of genes that are altered in human colon cancer (1, 2, 4–6, 8).

Most studies of gene expression in colon cancer have used as controls morphologically normal tissue from the same patient or group of patient (1, 3, 9). The assumption underlying this practice is that such tissue is also metabolically normal, with a gene expression profile identical to that of healthy colon mucosa. There are at least two reasons for questioning this assumption. First, in the $APC^{min}$ mouse (and in humans with familial adenomatous polyposis), multiple areas of the colon may develop polyps. Thus, before the appearance of polyps, any area in the colon may be in a precancerous state. Even in humans with no known hereditary risk for cancer, the emergence of multiple polyps or of a single cancer is associated with a greater risk for developing another cancer (18). Second, the formation of an adenomatous polyp or cancer may depend not only on genetic alterations within certain cells, but also on certain environmental events in the colon mucosa that may have a widespread distribution. Thus, one might expect that cancer would be associated with widespread metabolic changes in the colon.

**Differential Gene Expression in Adenomatous Polyps of $APC^{min}$ Mice.** Most of the genes we examined were regulated differently in polyps of $APC^{min}$ mice (Table 1). This is not surprising; human studies have reported differential regulation of hundreds of genes in adenomas as well as carcinomas of the colon (1, 9). However, the patterns of expression in the mice were somewhat different from those observed in human cancer. Most notably, several genes were up-regulated dramatically, at levels 80–400 times greater than in wild-type controls. Up-regulation to this degree has not been observed in human colon cancer. At least part of the difference may be attributable to use as a baseline colon mucosa from wild-type mice rather than from $APC^{min}$ mice, because expression of many genes was frequently higher in portions of the latter (Fig. 1).

These highly up-regulated genes have several functions. OPN, a ligand involved in cell signaling and communication, also affects inflammation and the response to cellular insults (19). Because of its role as a cell adhesion molecule, it is thought to facilitate metastasis. COX-2 is an inducible, rate-limiting enzyme that catalyzes the formation of prostaglandins from arachidonic acid. In addition to its involvement in the inflammation response, an increase in COX-2 expression is associated with many cancers, and COX-2 inhibitors are used as a cancer preventative treatment. Tumor growth has been reported to be decreased in double-knockout $APC^{min}$ mice in which COX-2 was also inactivated (20).

Of the other three gene products that demonstrated early up-regulation in $APC^{min}$ mice, two (MIP-2 and Gro-$\alpha$) are ligands for a third, the CXCR2 receptor. Gro-$\alpha$ is a known oncogene that has an autocrine action on CXCR2 to stimulate growth (21). The observation that three functionally related genes were all up-regulated raises the possibility that the initial event was up-regulation of one of these genes, followed by up-regulation of the others through compensatory mechanisms.

**Differential Gene Expression in Normal-Appearing Colon Mucosa of $APC^{min}$ Mice.** The five genes that were up-regulated to the highest degree in polyps—Gro-$\alpha$, OPN, MIP-2, CXCR2, and COX-2—were also up-regulated in polyp-free areas of the colons from these animals. In agreement with our reverse transcription-PCR results, we found that COX-2 activity was up-regulated in the grossly normal mucosa from $APC^{min}$ mice.

In mice that did have polyps, the location of the up-regulated areas was not correlated with the location of the polyps (Fig. 1); *i.e.*, the areas of greatest alteration of gene expression were just as likely to be distant from a polyp as close to it. This observation suggests that these areas of differential gene expression did not result from a field effect from a polyp but were independent loci. Because we did not examine this tissue under a microscope, we cannot rule out the possibility that it contained microscopic lesions such as ACF, which may have altered expression of certain genes (22). However, previous studies have reported that there are relatively few ACF in the colon of $APC^{min}$ mice (23, 24).

There are many cell types in the colonic mucosa, including epithelial cells, fibroblasts, and blood-borne cells. It is unlikely that the altered levels of gene expression that we observed can be attributed to

Table 3 *Gene expression levels in morphologically normal colon mucosa from human colon cancer patients as compared with noncancer subjects*

For Table 3A, colon mucosa samples were isolated from the sigmoidal-rectal region of noncancer subjects (78 samples from 12 individuals) and from the adjacent normal-appearing mucosa of patients with sigmoidal-rectal cancer (62 samples from 5 patients). For Table 3B, the colon mucosa samples were isolated from the ascending region of noncancer subjects (39 samples from 11 individuals) and from the adjacent normal-appearing mucosa of patients with ascending colon cancer (65 samples from 4 patients). Samples were analyzed for gene expression as described in the "Materials and Methods." Means ± SD are given for noncancer subjects, and ranges are given for cancer patients. Multivariate analysis was then performed on each gene taken in relation to all of the other genes to determine the significance of the difference between individuals with or without cancer.

| No. | Gene | Normal subjects (mean ± SD) | Cancer patients (range) | *P* |
|---|---|---|---|---|
| | | A. Sigmoidal-rectal colon | | |
| 1 | CXCR2[a] | 1.30 ± 1.11 | 0.81–210 | <0.01 |
| 2 | Gro-α | 2.93 ± 6.93 | 0.78–105 | NS[b] |
| 3 | IL-8 | 2.25 ± 2.63 | 1.22–82 | 0.0001 |
| 4 | COX-2 | 1.80 ± 2.63 | 0.91–66 | 0.001 |
| 5 | OPN | 1.55 ± 2.04 | 0.94–58 | 0.0001 |
| 6 | Gro-γ | 1.92 ± 3.34 | 0.80–37 | NS |
| 7 | MCSF-1 | 1.54 ± 1.40 | 1.54–31 | 0.0001 |
| 8 | COX-1 | 1.22 ± 0.87 | 0.12–9.58 | NS |
| 9 | CD44 | 1.12 ± 0.56 | 0.54–6.52 | <0.05 |
| 10 | c-myc | 1.24 ± 0.82 | 0.12–4.76 | NS |
| 11 | Cyclin D | 1.28 ± 0.84 | 0.43–4.44 | NS |
| 12 | PPAR-α | 1.10 ± 0.62 | 0.02–2.87 | NS |
| 13 | PPAR-δ | 1.15 ± 0.55 | 0.02–1.90 | <0.01 |
| 14 | p21 | 1.04 ± 0.29 | 0.40–1.68 | <0.01 |
| 15 | PPAR-γ | 1.07 ± 0.40 | 0.01–1.28 | <0.01 |
| | | B. Ascending colon | | |
| 1 | CXCR2 | 1.32 ± 1.08 | 1.90–90 | <0.05 |
| 2 | Gro-α | 1.60 ± 2.08 | 0.46–30 | NS |
| 3 | IL-8 | 1.66 ± 1.62 | 1.32–183 | <0.05 |
| 4 | COX-2 | 1.84 ± 3.04 | 2.96–153 | 0.0001 |
| 5 | OPN | 1.53 ± 1.31 | 9.24–153 | 0.0001 |
| 6 | Gro-γ | 1.40 ± 1.41 | 0.63–11 | NS |
| 7 | MCSF-1 | 1.68 ± 1.62 | 4.01–40 | 0.0001 |
| 8 | COX-1 | 1.17 ± 0.75 | 0.84–45 | <0.001 |
| 9 | CD44 | 1.11 ± 0.51 | 0.99–14 | 0.0001 |
| 10 | c-myc | 1.16 ± 0.63 | 0.39–10.82 | NS |
| 11 | Cyclin D | 1.38 ± 1.08 | 0.12–13.15 | NS |
| 12 | PPAR-α | 1.16 ± 0.58 | 0.22–4.09 | NS |
| 13 | PPAR-δ | 1.13 ± 0.55 | 0.02–7.08 | <0.05 |
| 14 | p21 | 1.09 ± 0.40 | 0.04–2.66 | NS |
| 15 | PPAR-γ | 1.08 ± 0.42 | 0.01–1.14 | 0.01 |

[a] CXCR2, CXC cytokine receptor 2; Gro-α and -γ, growth-related oncogene-α and -γ; IL-8, interleukin-8; COX-1 and -2, cyclooxygenase-1 and -2; OPN, osteopontin; MCSF-1, macrophage-colony-stimulating factor; PPAR-α, -δ, and -γ, proliferating peroxisome-activating receptor-α, -δ, and -γ.

[b] NS, not significant at $P < 0.05$ level.

alterations localized to ACF alone. Indeed, COX-2 may not be expressed in the colonic epithelial cells; it is expressed in interstitial cells at an early stage (25). There are some reports of expression of COX-2 in stromal macrophages in tumor tissues (26–28). Elevated expression levels of COX-2 were also demonstrated in macrophages in the lamina propria of histologically normal epithelium of $APC^{min}$ mice but not in the mucosa of wild-type mice (26). In agreement with the literature, we also observed prominent expression of COX-2 in macrophages but only weak COX-2 staining in epithelial cells of ACF. Mucin secreted by colon cancer cells can induce the expression of COX-2 in macrophages located in the surrounding area, and it has been suggested that activated macrophages may provide a favorable microenvironment for epithelial cell growth (28).

We were unable to detect immunohistochemical staining of Gro-α and OPN, perhaps because both are secreted, unstable proteins and therefore evanescent in tissue sections. In addition, the $C_T$ values obtained from quantitative reverse transcription-PCR indicates that the absolute expression level of CXCR2 was low, which may explain why we could not demonstrate CXCR2 staining. If the absolute RNA quantities are sufficient, RNA *in situ* hybridization may be a better method to determine the cellular locations of up-regulation of these genes. Regardless of the cell types responsible for the overexpression of these genes, our results demonstrate that the RNA expression level is a more sensitive indicator of abnormal colon mucosa than cytological or architectural changes.

**Differential Gene Expression in Normal-Appearing Colon Mucosa of Human Cancer Patients.** As with $APC^{min}$ mice, several of the same genes were significantly up-regulated, and several other genes were significantly down-regulated, in the morphologically normal mucosa from patients with colon cancer (Table 3). These alterations were not restricted to certain regions of colon mucosa because they occurred in patients with ascending colon carcinomas or sigmoidal-rectal carcinomas and most of the genes involved were similar in the two regions. However, there was considerable variability in expression level from one area of the colon to another, and these differences did not correlate with distance from the tumor. Thus, as with the mice, these metabolic alterations in normal-appearing colon mucosa are apparently not the consequence of a field effect.

**Are Metabolic Disturbances Related to Carcinogenesis?** Our findings in $APC^{min}$ mice and human colon cancer patients raise the

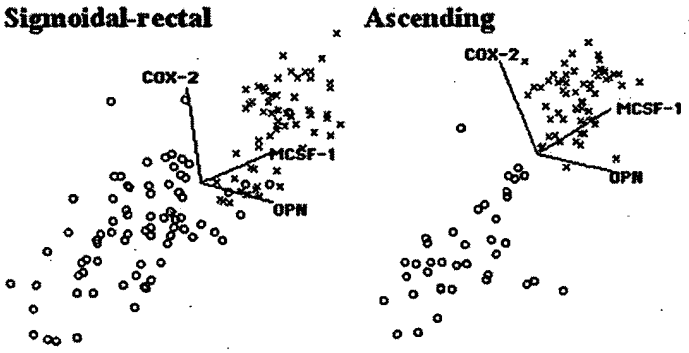


Fig. 4. Three-gene expression analysis of normal-appearing colon mucosa in patients with cancer in sigmoidal-rectal colon and ascending colon. Mucosa samples were taken from each colon cancer patient (see Table 3 for number of patients and number of samples/patient). Biopsies were also taken from noncancer patients (per subject from sigmoidal-rectal area and per subject from ascending colon). Each sample was analyzed for expression levels of cyclooxygenase-2 (*COX-2*), osteopontin (*OPN*), and macrophage-colony-stimulating factor (*MCSF-1*), and the values are plotted on three perpendicular axes. ✕, values for a cancer patient; ○, values for a noncancer patient. *Left*, sigmoidal-rectal colon; *right*, ascending colon.

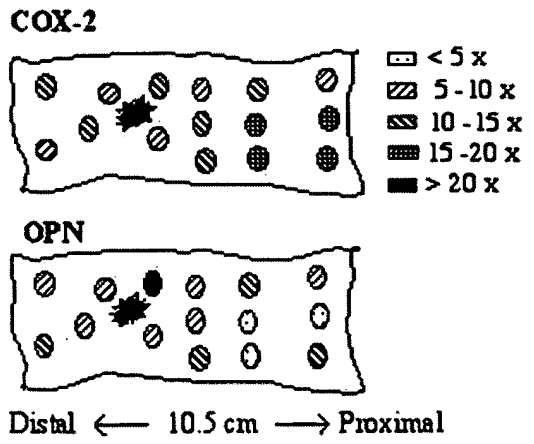


Fig. 5. Distribution of cyclooxygenase-2 (*COX-2*) and osteopontin (*OPN*) expression in sigmoidal-rectal colon of a single cancer patient. The patient had a cancer in the sigmoidal-rectal colon, as indicated by the *spot* with *jagged edges*. Locations of mucosa samples removed for analysis are indicated by the *stippled*, *hatched* and *meshed circles*. Levels of COX-2 and OPN expression in each sample were determined by reverse transcription-PCR and are indicated approximately by various patterns as shown. The mean levels of expression of COX-2 and OPN in colon mucosa of non-cancer patients were 1.80 and 1.55, respectively (as shown in Table 3).

question of what defines a "normal" colon. Although expression levels of many of the genes we examined were much higher in polyps and cancer than apparently normal mucosa in surrounding tissue, expression levels in the apparently normal mucosa were significantly higher than those in controls, indicating that this surrounding tissue is not metabolically normal. Use of such tissue as a control or baseline in gene expression studies for carcinoma is likely to underestimate the degree of altered regulation of certain genes.

What is the meaning of these metabolic alterations in morphologically normal tissue? Given that these alterations are more marked in polyps and cancer, we speculate that these changes may indicate a predisposition to develop cancer. For example, overexpression of COX-2 in mice increased the likelihood that cells would be transformed after exposure to mutagens (29). Thus, our observation of gene up-regulation in morphologically normal colon mucosa suggests that these cells may be abnormally sensitive to events that do not affect metabolically normal mucosa.

Some of the genes up-regulated in the normal mucosa from cancer patients are involved in inflammation. These include not only COX-2, but also cytokines and chemokines such as Gro-$\alpha$, MCSF-1, IL-8, and MIP-2. Many cancers arise from sites of infection, chronic irritation, or inflammation (30), and COX-2 inhibition may be a useful chemopreventive agent for colon cancer (31). The other genes we have identified that have altered expression before adenomatous polyp formation may also be useful targets for cancer prevention as well as early markers for cancer screening and diagnosis.

Regardless of the underlying mechanisms, our finding of metabolic changes in normal-appearing colon mucosa of cancer patients suggests that analysis of this tissue, through a rectal smear test or lavage, for example, might be used as a simple, noninvasive screening test for colon cancer. Although a significantly higher or lower level of expression of a single gene might not be a very reliable indicator, our multivariate analysis suggests that analysis of a group of genes might provide a much better indicator of increased risk.

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